University of Alberta

Inflammation, muscle wasting and amino acid supplementation in the tumor-bearing state

by

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ABSTRACT

Cachexia is common in cancer and is characterized by skeletal muscle wasting. The hypothesis of this thesis is that inflammation contributes to the loss of body weight and skeletal muscle in the tumor-bearing state through an increase in the metabolic demand for specific amino acids, specifically cysteine. Cysteine is suggested to be conditionally essential during inflammation through an increase in hepatic utilization that limits cysteine availability for peripheral tissue. To determine the role of inflammation on skeletal muscle wasting in the tumor-bearing state, the severity of inflammation was enhanced by endotoxin administration and suppressed by ibuprofen treatment. The Yoshida hepatoma increased skeletal muscle proteolysis by 38% ($p=0.003$) and the administration of endotoxin increased proteolysis by an additional 18% ($p=0.03$). The low dose of endotoxin used did not affect skeletal muscle protein metabolism in non-tumor-bearing rats; therefore it was concluded that skeletal muscle was sensitized to the catabolic effects of acute stimulation by the presence of a tumor. Ibuprofen treatment in mice bearing the colon 26 tumor reduced the loss in body weight ($p=0.01$) and skeletal muscle mass ($p=0.02$) with a partial restoration of skeletal muscle protein synthesis ($p=0.03$), tissue glutathione ($p=0.01$ for liver and $p=0.04$ for muscle) and plasma cyst(e)ine ($p=0.03$) compared to untreated tumor-bearing mice at an equal tumor burden. Dietary supplementation with cystine did not improve skeletal muscle mass or protein synthesis in colon 26-bearing mice; however, plasma and muscle cyst(e)ine concentration was also not increased with supplementation. Although cystine

supplementation increased tumor cyst(e)ine concentration ($p=0.009$), tumor growth and protein synthesis were not affected. Cystine supplementation also reversed the positive effects of ibuprofen on body weight and skeletal muscle mass but increased the tumor response to treatment with the chemotherapeutic agent, irinotecan ($p=0.01$). The studies of this thesis have established the specific role of inflammation on skeletal muscle loss in the tumor-bearing state independent of the effect of inflammation on tumor progression. The data suggest that the use of non-steroidal anti-inflammatory agents may be effective in the treatment of cachexia. The exaggerated metabolic response to acute inflammatory stimulation in the tumor-bearing state suggests that infections can contribute substantially to cachexia in patients with cancer.

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ABBREVIATIONS

BCAA: Branched chain amino acids

BCAAT: Branched chain amino acid transferase

C26: Colon 26 adenocarcinoma

CON: Control

COX: Cyclooxygenase

CPT-11: Irinotecan (Camptosar®)

CRP: C-reactive protein

Cys: Cystine

Cyst(e)ine: Cysteine and/or cystine

DPM: Disintegrations per minute

HPLC: High performance liquid chromatography

IAAO: Indicator amino acid oxidation

Ibu: Ibuprofen

IL-1β: Interleukin-1beta

IL-6: Interleukin-6

iNOS: Inducible nitric oxide synthase

LPS: Lipopolysaccharide (endotoxin)

N: Nitrogen

NF-κB: Nuclear factor-kappaB

OPA: Ortho-phthaldialdehyde

PCA: Perchloric acid

PGE_2: Prostaglandin E_2

REE: Resting energy expenditure

Ref: Reference

ROS: Reactive oxygen species

SA: Specific activity

SAA: Sulphur amino acids

TCA: Trichloroacetic acid

TNFα: Tumor necrosis factor-alpha

YAH: Yoshida ascites hepatoma 130

CHAPTER 1. BACKGROUND AND RATIONALE

1.1 OVERVIEW

1.1.1 CACHEXIA IS PREVALENT AND ASSOCIATED WITH POOR OUTCOME IN CANCER

Cachexia is characterized by a loss of body weight and muscle wasting associated with underlying disease (i.e. cancer, HIV/AIDS, chronic obstructive pulmonary disease, chronic heart failure) (Baracos, 2006; MacDonald et al., 2003). The prevalence of cachexia in cancer varies by disease site, but overall, half the patients with cancer have weight loss at diagnosis (Dewys et al., 1980). The prevalence of weight loss increases during the trajectory of cancer from diagnosis through anti-tumor therapy to palliation (Sarhill et al., 2003). Cancer cachexia is associated with poor outcomes in regards to chemotherapy response and survival (Andreyev et al., 1998; Dewys et al., 1980; Hauser et al., 2006; Ross et al., 2004); therefore, it is essential that mechanisms of cachexia are identified so that appropriate therapies can be developed.

1.1.2 ALTERED PROTEIN METABOLISM IN CANCER CACHEXIA RESULTS IN THE LOSS OF MUSCLE MASS

The hormonal and metabolic abnormalities in cancer cachexia result in a hypermetabolic state with a net loss of body tissue. Altered protein metabolism is particularly evident with elevations in whole-body protein turnover and loss of lean body mass, specifically skeletal muscle mass. The loss of skeletal muscle mass results from an imbalance in skeletal muscle protein turnover where anabolism is reduced while catabolism is often elevated (Dworzak et al., 1998; Emery et al., 1984; Khal et al., 2005; Lundholm et al., 1976; Williams et al., 1999). Despite the increased risk of morbidity and mortality associated with the loss of lean body mass (Demling, 2007), there are no specific recommendations for the optimal protein nutrition that will support protein anabolism in cancer due to an insufficient understanding of amino acid requirements and the underlying basis of altered amino acid utilization in this setting.

1.1.3 Does inflammation cause muscle wasting in cancer cachexia?

Inflammation is a common element in several wasting diseases (Delano & Moldawer, 2006) and is associated with cachexia in cancer (Barber et al., 1999; Scott et al., 2002; Staal-van den Brekel et al., 1995). Although inflammation is an integral component of the innate immune response, chronic activation can result in utilization of body nutrient reserves. Pro-inflammatory mediators promote the production of proteins involved in the acute-phase response and the loss of skeletal muscle mass (Argiles et al., 2005). During inflammation there is a redirection of amino acid supply from peripheral tissue to visceral tissue and an increased utilization of some amino acids that might limit amino acid availability for skeletal muscle (Obled et al., 2002). The enhanced production of proteins involved in the acute-phase response and the tri-peptide antioxidant glutathione increase the utilization of cysteine during inflammation (Grimble, 1990; Malmezat et al., 2000a). Although cysteine is not a dietary essential amino acid, *de novo* synthesis may not be adequate to support the increased demand for cysteine. The addition of cysteine to an amino acid mixture increased skeletal muscle mass and reduced nitrogen loss in a rodent model of acute inflammation, suggesting that cysteine is conditionally essential (Breuille et al., 2006). Although body weight and body cell mass are increased by the provision of N-acetyl-cysteine or a high cysteine protein in cancer patients (Hack et al., 1998; Tozer et al., 2008), the specific conjecture that cysteine is conditionally essential in the tumor-bearing state has not been tested.

The following review of the literature examines the metabolic alterations associated with the development of cachexia, the evidence for the role of inflammation in skeletal muscle wasting in cancer, and how inflammation might impact the amino acids required in cancer, with the emphasis on cysteine. The final section provides the hypothesis and scope of the thesis, as well as the specific hypotheses and objectives for each study that this thesis encompasses.

1.2 Cancer cachexia

1.2.1 Cachexia prevalence and outcomes in cancer

Cachexia is common in cancer, as demonstrated by the prevalence of weight loss at diagnosis. The seminal study examining weight loss in cancer patients prior to receiving chemotherapy treatment was published by Dewys et al. (1980). Over half (54%) of 3047 patients with cancer experienced weight loss in the previous six month period. The prevalence of weight loss varied among the types of cancer. Patients with non-Hodgkin's lymphoma, acute nonlymphocytic leukemia or breast cancer had the lowest frequency of weight loss (31-36%). In contrast, 54-59% of patients with colon or lung cancer had weight loss. More recent studies on weight loss at diagnosis in patients with cancer have demonstrated similar results. Of the 151 patients with newly diagnosed gastrointestinal (half with colorectal cancer) or lung cancer, 58% had lost body weight in the previous six months (Khalid et al., 2007). Compared to the prevalence at diagnosis, weight loss was more common in patients with advanced cancer admitted to a palliative program, where 85% of patients experienced some weight loss, and one third lost more than 20% of body weight in the previous 6 months (Sarhill et al., 2003).

Weight loss in cancer has been associated with poor prognosis, and Dewys et al. (1980) showed that weight loss greater than 5% in the 6 months before chemotherapy was associated with a reduction in chemotherapy response and shortened survival. A recent review of 14 studies (n=9,527) that examined weight loss with cancer concluded that weight loss is a prognostic factor for shortened survival (Hauser et al., 2006). Patients with non small-cell lung or gastrointestinal cancer who had weight loss before chemotherapy were more likely to not complete the treatment protocol due to toxicity symptoms; they also had reduced survival compared to patients without weight loss (Andreyev et al., 1998; Ross et al., 2004). A factor in the increased toxicity of chemotherapy may have been related specifically to the loss of lean body mass. Patients with colorectal cancer and low lean body mass were more likely to experience

toxicity related to chemotherapy (Prado et al., 2007). The prevalence and negative outcomes associated with cancer cachexia warrant investigation into potential treatments to alleviate weight and muscle loss.

1.2.2 Protein metabolism and skeletal muscle wasting in cancer cachexia

Metabolic alterations in cancer could increase nutrient utilization and result in a net catabolic state. Whole-body protein turnover rate in patients with lung, colon or gastric cancer was higher than that in healthy controls (Dworzak et al., 1998; Fearon et al., 1988; Heber et al., 1982; Jeevanandam et al., 1984; Melville et al., 1990). Although not universal, some patients with cancer have an elevated rate of resting energy expenditure (REE). Compared to non-cancer controls, REE was increased in patients with lung or colon cancer (Jatoi et al., 2001; Staal-van den Brekel et al., 1997), whereas others have found no difference (Fearon et al., 1988). However, the comparison of measured to expected REE in almost 300 patients with cancer indicated that almost half were classified as hypermetabolic while only 2% of subjects were classified as hypometabolic (Bosaeus et al., 2001). An elevated REE is not associated with an increase in dietary intake in patients with cancer (Bosaeus et al., 2001). If energy intake is not adequate to support energy expenditure, body nutrient reserves would be utilized resulting in the loss of body weight.

The loss of muscle mass occurs when there is an imbalance between protein synthesis and protein degradation. Stable isotope studies in patients with cancer and weight loss have demonstrated a reduction in skeletal muscle protein synthesis (Dworzak et al., 1998; Emery et al., 1984; Lundholm et al., 1976) and an increase in muscle protein degradation through activation of the ubiquitin-proteasome pathway (Khal et al., 2005; Williams et al., 1999). Expression of the ubiquitin-proteasome pathway components was higher in skeletal muscle from patients with cancer only when weight loss was greater than 10% of body weight (Khal et al., 2005), suggesting that another proteolytic pathway is responsible for early muscle loss. Patients with early-stage lung

cancer and minor weight loss had increased mRNA levels for cathepsin B in skeletal muscle (Jagoe et al., 2002). Thus, lysosomal proteolysis may have a role in early muscle wasting, followed by activation of the ubiquitin-proteasome pathway.

The loss of skeletal muscle mass is an important factor in the association between weight loss and prognosis. Studies in elderly men and women have shown an increase risk for physical disability when skeletal muscle mass index is low (Baumgartner et al., 1998; Janssen et al., 2004). Also, the loss of lean body mass is associated with increased hospitalization, impaired immune function, infections, delayed wound repair and mortality (Demling, 2007). Therefore, adequate protein nutrition to support protein anabolism in cancer is required to reduce the risks associated with a loss of skeletal muscle mass. Recent attention to the provision of specific amino acids or proteins in cancer (May et al., 2002; Tozer et al., 2008) is based on the concept that skeletal muscle protein synthesis is limited by reduced availability of specific amino acids. The application of amino acid and protein supplements in cancer is currently done without a complete understanding of the factors that influence amino acid utilization and requirements. An association between an inflammatory response in patients with cancer and metabolic alterations, including increased REE (Falconer et al., 1994; Scott et al., 2001; Staal-van den Brekel et al., 1995; Wigmore et al., 1997), whole-body protein turnover (Fearon et al., 1991) and increased hepatic synthesis of acute-phase proteins (Fearon et al., 1998; Preston et al., 1998), suggests that inflammation is a factor in the development of cachexia. The following sections will examine the inflammatory response and how inflammation relates to skeletal muscle wasting and modified amino acid utilization.

1.3 THE RELATIONSHIP BETWEEN INFLAMMATION AND SKELETAL MUSCLE WASTING

1.3.1 MEDIATORS OF INFLAMMATION

Inflammation is a part of the innate immune system that provides an immediate response to injury or pathogens and serves a critical role in survival by aiding in tissue protection and repair (Baumann & Gauldie, 1994; Gabay & Kushner, 1999). Although vital in acute situations, chronic inflammation can result in tissue damage and wasting of body tissues. Mediators of inflammation, include the eicosanoids, pro-inflammatory cytokines and nitric oxide. These mediators are regulated by nuclear factor-kappaB (NF-κB) transcription factor (Li & Verma, 2002). Prostaglandins and leukotrienes are eicosanoids produced from cyclooxygenase (COX). Although eicosanoids have an essential role in normal cell function, those produced from the inducible COX isoform (COX-2) are involved in mediating the inflammatory response, including the promotion of vasodilatation, platelet aggregation, fever, and pain (Funk, 2001). The pro-inflammatory cytokines that initiate and promote the acute phase response include tumor necrosis factorα (TNFα), interleukin-1β (IL-1β) and interleukin-6 (IL-6). Macrophages and monocytes at the site of inflammation initially produce pro-inflammatory cytokines, but other cell types also produce cytokines. Following the administration of a non-lethal dose of endotoxin, the mRNA expression of TNFα and Il-1β is increased in liver, spleen, kidney, heart and skeletal muscle, followed by increased IL-6 mRNA expression (Lang et al., 2003). Cytokines can act in a paracrine, autocrine or endocrine manner to stimulate and activate cells of the immune system, including T–lymphocytes, B-lymhocytes, natural killer cells, monocytes and macrophages (Curfs et al., 1997). Pro-inflammatory cytokines also induce fever, promote endothelial cell adhesion and stimulate synthesis of acute-phase plasma proteins (Curfs et al., 1997).

Acute-phase proteins produced during inflammation include C-reactive protein (CRP), fibrinogen, α1-acid glycoprotein, haptoglobin, serum amyloid A and ceruloplasmin. The increase in plasma concentration of acute-phase proteins

can be as high as 1000-fold for CRP, with others such as haptoglobin and fibrinogen increasing 2 to 4 fold (Gabay & Kushner, 1999). In contrast to the acute-phase proteins, the concentrations of other plasma proteins, such as albumin, are reduced during inflammation. Even though plasma albumin concentration is lower during inflammation, albumin synthesis is often not reduced (Fearon et al., 1998; Jahoor et al., 1999; Mackenzie et al., 2003; Mansoor et al., 1997), suggesting an increase in degradation or loss from vascular circulation. Considering that albumin synthesis accounts for 8% of whole-body protein synthesis in the non-pathological state (De Feo et al., 1992), elevated rates of albumin synthesis would increase the metabolic demand for amino acids.

1.3.2 INFLAMMATORY MEDIATORS PROMOTE THE LOSS OF SKELETAL MUSCLE PROTEIN

Pro-inflammatory cytokines have a direct effect of skeletal muscle protein metabolism. Cytokine receptor mRNAs are expressed in skeletal muscle and are upregulated in response to *in vivo* administration of endotoxin or incubation with TNFα *in vitro* (Zhang et al., 2000), which indicates that skeletal muscle is responsive to the effects of cytokines. The pro-inflammatory cytokines increase skeletal muscle proteolysis primarily through activation of the non-lysosomal ubiquitin-proteasome pathway, the main pathway for degradation of cytosolic proteins. The ubiquitin-proteasome pathway has been implicated in several states of muscle wasting (Lecker et al., 1999; Lecker, 2003). The first two enzymes of the ubiquitin-proteasome pathway activate and transfer ubiquitin to the third enzyme, ubiquitin ligase, which attaches ubiquitin to proteins. The ubiquitin ligase is responsible for recognizing and targeting proteins for degradation by the proteasome. Several types of muscle-specific ubiquitin ligases have been identified, and at least three have been found to have increased expression in states of muscle atrophy (Bodine et al., 2001; Kwak et al., 2004; Lecker et al., 2004). Administration of TNFα in rats or incubation medium increased the expression of ubquitin mRNA in skeletal muscle (Garcia-Martinez et al., 1995; Llovera et al., 1997; Llovera et al., 1998). Moreover,

incubation of muscle cells with either IL-6 or TNFα increased the expression of mRNA of a muscle-specific ubiquitin ligase, E3α-II (Kwak et al., 2004).

Although, cytokines have a direct effect on skeletal muscle proteolysis, the actions of inflammatory mediators on skeletal muscle protein synthesis are less clear. Chronic abdominal sepsis or acute inflammation induced by endotoxin in rats lowered skeletal muscle protein synthesis through a reduction in translation efficacy (Cooney et al., 1994; Cooney et al., 1999; Lang et al., 2000; Vary et al., 1996a; Vary et al., 1996b). An alteration in regulatory initiation factors was determined to be the mechanism involved in the reduction in translational efficacy (Lang et al., 2000; Vary et al., 1996b). A reduction in skeletal muscle insulin-like growth factor-1 occurred during chronic abdominal sepsis or acute inflammation induced by endotoxin; therefore, a reduction in insulin-like growth factor-1 may be responsible for lower protein synthesis rather than a direct effect of cytokines (Lang et al., 1996; Lang et al., 2000; Lang et al., 2002). Inflammatory mediators can also indirectly limit skeletal muscle protein synthesis through a redistribution of amino acid supply and an increased utilization of particular amino acids. During inflammation, a redistribution of amino acid supply from peripheral tissue to the liver occurs (Breuille et al., 2006). Although all amino acids are required for the synthesis of proteins, the utilization of certain amino acids may be increased during inflammation based on the amino acid composition of proteins involved in the acute-phase response (Grimble, 1990; Reeds et al., 1994). Since protein synthesis is dependent on the availability of the correct amount of each amino acid, an inadequate level of any one of the amino acids required for muscle protein would limit skeletal muscle protein synthesis. Also, if the exogenous supply of amino acids is inadequate and skeletal muscle catabolism is relied upon to supply the amino acids, excessive muscle protein would need to be degraded to obtain the required amino acids (and the remaining amino acids catabolized) because the amino acid composition of skeletal muscle differs from that of acute phase proteins (Barker & Putnam, 1984; Reeds et al., 1994).

Supplementation of specific amino acids during acute inflammation has shown positive results on skeletal muscle mass. Rats provided with supplemental cysteine, taurine, threonine and serine had higher body weight and lower 3-methylhistidine excretion following turpentine-induced inflammation compared to controls provided with a standard amino acid formulation (Osowska et al., 2003). The addition of glutamine to the supplemental amino acid mixture also improved nitrogen content of skeletal muscle and liver (Osowska et al., 2003). A mixture of threonine, serine, aspartate, asparagine, arginine and cysteine increased skeletal muscle mass and reduced nitrogen loss in rats injected with live bacteria compared to controls provided additional alanine (Breuille et al., 2006). These amino acid supplementation studies suggest that amino acid requirements are altered during inflammation, and some amino acids become conditionally essential. The magnitude and nature of inflammation would be expected to influence the metabolic response; therefore, it is unclear if inflammation associated with cancer has an impact on skeletal muscle mass and amino acid requirements similar to that occurring with acute inflammation.

1.3.3 Role of chronic inflammation associated with cancer in skeletal muscle wasting and altered amino acid requirements

Inflammation in cancer, as indicated by elevated CRP or serum IL-6, is associated with a reduction in body weight, lean body mass, performance status and survival in patients with pancreatic, lung or colorectal cancer (Al-Shaiba et al., 2004; Barber et al., 1999; Belluco et al., 2000; Canna et al., 2004; Chung & Chang, 2003; Crozier et al., 2006; Falconer et al., 1995; Forrest et al., 2003; McMillan et al., 2003; Read et al., 2006; Scott et al., 2002; Staal-van den Brekel et al., 1995; Wong et al., 2007). The role of inflammation in cancer is also suggested by studies with nonspecific COX inhibitors. These agents reduced acute-phase protein concentration, REE and whole-body protein turnover; and increased body weight and mid-arm circumference in cancer patients (Lundholm et al., 1994; Lundholm et al., 2004; McMillan et al., 1995;

McMillan et al., 1999; Preston et al., 1995; Wigmore et al., 1995). Also, long-term use of a nonspecific COX inhibitor was associated with prolonged survival in patients with cancer (Lundholm et al., 1994). However, the independent effect of inflammation in cancer on lean body mass and survival is unclear, because inflammation is associated with disease progression. Serum CRP and IL-6 are associated with tumor stage, metastases and tumor markers in colorectal cancer (Belluco et al., 2000; Chung & Chang, 2003). The link between inflammation and disease progression in cancer is attributed to the role of inflammatory mediators in tumor growth. The production of pro-inflammatory cytokines and prostaglandins by tumor cells and tumor-associated macrophages promote cell proliferation, angiogenesis, tissue evasion, and metastases, but inhibit apoptosis (Balkwill & Mantovani, 2001; O'Byrne & Dalgleish, 2001; Zitvogel et al., 2006).

Treatment of mice bearing the colon 26 (C26) adenocarcinoma with COX inhibitors increased muscle mass and reduced serum IL-6 and spleen NF-κB activation (Davis et al., 2004; Fujimoto-Ouchi et al., 1995; Zhou et al., 2003). The increase in skeletal muscle mass resulting from treatment with COX inhibitors in another tumor model, the Yoshida ascities hepatoma (YAH), occurred through an elevation in protein synthesis and a reduction in net protein degradation (Strelkov et al., 1989). However, in both the C26 and YAH models, tumor growth was suppressed by treatment with COX inhibitors (Davis et al., 2004; Fujimoto-Ouchi et al., 1995; Strelkov et al., 1989; Zhou et al., 2003). Therefore, the association between inflammation and skeletal muscle wasting in the tumor-bearing state has not been evaluated independent of tumor burden.

1.4 Inflammation and cysteine requirement: rationale for investigation of cysteine in the tumor-bearing state

1.4.1 Cysteine utilization is increased during acute inflammation

Aspects of cysteine utilization and supply during acute inflammation suggest that cysteine could become conditionally essential. The bulk of cysteine

utilization is for the synthesis of protein and glutathione, with each accounting for 40-50% of cysteine flux in the non-pathological state (Fukagawa et al., 1996). During inflammation cysteine utilization is elevated to support the production of glutathione, as well as proteins involved in the acute-phase response (Grimble, 1990; Obled et al., 2002). The incorporation of cysteine into plasma proteins other than albumin, which includes the positive acute-phase proteins, is increased 70% following a bacterial injection in rats (Malmezat et al., 1998).

Albumin synthesis during inflammation might also be quantitatively important. Even though plasma albumin concentrations may be lower during inflammation, albumin synthesis is often not reduced or even increased (Fearon et al., 1998; Mackenzie et al., 2003; Mansoor et al., 1997). Since albumin contains a relatively high amount of cysteine at 6.4% (Barker & Putnam, 1984), an increase in synthesis would have an impact on cysteine demand. Pancreatic cancer patients with an inflammatory response demonstrated an increase in albumin synthesis of 50mg/kg/day (Fearon et al., 1998) which equates to an additional 26μmol cysteine/kg/day.

In addition to plasma proteins, cysteine incorporation into other tissue such as spleen and lung is elevated in the rat model of acute inflammation, although cysteine incorporation into skeletal muscle protein is reduced (Malmezat et al., 1998). Intracellular metallothioneins protect cells against DNA damage from reactive oxygen species, and are a part of the acute-phase response (Fischer & Davie, 1998; Nath et al., 2000). Metallothionein was among the most strongly induced mRNA (up to 20-fold) in microarray analysis of rat muscles with atrophy due to different causes, including YAH model (Lecker et al., 2004). Since one third of metallothionein amino acid composition is cysteine, increased production of this protein would be expected to have a quantitative impact on cysteine demand.

Cysteine utilization to support glutathione synthesis likely has a larger role than cysteine utilization to support acute-phase protein synthesis during inflammation. Glutathione (γ-glutamyl-cysteinyl-glycine) is the most abundant intracellular thiol (Townsend et al., 2003). Glutathione has several functions including scavenging of reactive oxygen species, detoxification of xenobiotics and formaldehyde, synthesis of eicosanoids, and protein thiolation (Townsend et al., 2003). Acute inflammation in rats increased the absolute glutathione synthesis rate by 236% in 8 different tissues (Malmezat et al., 2000a). Incubation of rat hepatocytes with TNFα increased glutathione synthesis through an increase in γ-glutamyl-cysteine synthetase (Morales et al., 1997). The importance of cysteine intake in maintaining glutathione levels during inflammatory stress has been demonstrated. Following administration of TNFα, rats fed a low protein diet can maintain similar glutathione concentrations similar to those of rats fed a high protein diet, but only if cysteine is supplemented in the diet (Grimble et al., 1992; Hunter & Grimble, 1994; Hunter & Grimble, 1997). Therefore, adequacy of cysteine supply is the main determinate of glutathione levels during periods of acute inflammatory stress.

Cysteine is supplied from the diet, from protein/glutathione breakdown, and from biosynthesis from methionine and serine. A lower dietary intake in general and/or the consumption of proteins that are low in sulphur amino acids can reduce cysteine and methionine supply during inflammation. Low food intakes are endemic in disease conditions including cancer (Bosaeus et al., 2001; Hutton et al., 2006), and many enteral nutrition support supplements are based on casein, which contains very little cysteine (0.35%). Methionine transulphuration is increased 2-fold during acute inflammation (Malmezat et al., 2000b); however, since the transulphuration pathway accounts for only 5-14% of cysteine flux (Fukagawa et al., 1996), *de novo* synthesis of cysteine may not be sufficient to meet the increased metabolic demand. Without an increase in cysteine supply, the increased utilization of cysteine during inflammation could reduce cysteine availability for skeletal muscle.

Cysteine has been identified as the most limiting amino acid in a rat model of acute inflammation. Rats administered live bacteria were supplemented with serine, threonine, aspartate, asparagine and arginine with the addition cysteine at multiple levels for 7 days before and 10 days after the challenge (Breuille et al., 2006). The mixture with the highest level of cysteine increased skeletal muscle mass and reduced nitrogen loss (Breuille et al., 2006).

1.4.2 Is cysteine conditionally essential in cancer?

The literature on cysteine supplementation during acute inflammation suggests that cysteine could be conditionally essential in cancer. However, the degree of cysteine utilization during inflammation associated with tumor growth is not known. Plasma concentrations of cyst(e)ine are lowered in chronic inflammatory conditions such as cancer (Droge & Holm, 1997). Furthermore, skeletal muscle glutathione is decreased in tumor-bearing mice (Hack et al., 1996). Cysteine administered intraperitoneally to tumor-bearing mice increased hepatic sulfate levels and muscle glutathione levels (Hack et al., 1996; Ushmorov et al., 1999), suggesting that cysteine availability is reduced in the tumor-bearing state. Long term oral N-acetyl-cysteine administration in patients with advanced cancer increased body cell mass and plasma albumin (Hack et al., 1998). Also, cancer patients supplemented with a high cysteine whey protein had increased body cell mass and body weight (Tozer et al., 2008). However, none of the studies in the tumor-bearing state examined the effect of cysteine on skeletal muscle mass and protein metabolism.

1.5 Rationale and scope of thesis

Cancer cachexia is a prevalent and debilitating condition that is associated with negative outcomes including impaired chemotherapy response and shortened survival. Metabolic alterations in cachexia result in the loss of lean tissue through an increase in catabolism and a reduction in anabolism. Since the loss of lean body mass results in impaired function, it is necessary to develop

nutritional therapies that will prevent or treat lean body mass in cancer. However, the current understanding of amino acid requirements in cancer is insufficient to support recommendations for protein nutrition. Although inflammation in cancer is associated with cachexia, the independent impact of inflammation on skeletal muscle wasting is not clear due to the relationship between tumor progression and inflammation. Moreover, the role of inflammation on the metabolic demand for amino acids in cancer is not known. Cysteine is suggested to become conditionally essential during inflammation; however, it has not been determined if cysteine is conditionally essential in the tumor-bearing state.

The objectives of this thesis were to:

1.) critically review the literature on the utilization of amino acids in the tumor-bearing state,
2.) establish the specific role of inflammation in the tumor bearing state on the loss of skeletal muscle mass , and
3.) determine if a demand for cysteine is driven by inflammation in the tumor-bearing state.

The hypothesis of this thesis is that inflammation contributes to the loss of body weight and skeletal muscle in the tumor-bearing state through an increase in the metabolic demand for amino acids, specifically cysteine. This thesis is composed of one critical review of the literature pertaining to amino acid metabolism and supplementation in tumor-bearing models (***Chapter 2***) and three separate studies with the following specific hypotheses and objectives related to the overall scope of the thesis:

Chapter 3 Main Theme: Potentiation of muscle wasting by inflammation in the tumor-bearing state.

The role of inflammation in cancer-associated skeletal muscle loss has not been fully determined. Therefore, an acute inflammatory stimulus was used to

augment inflammation in Yoshida ascites hepatoma (YAH)-bearing rats. YAH produced a rapid loss of weight and skeletal muscle associated with an increase in systemic TNFα and prostaglandin E_2 (Baracos et al., 1995; Busquets et al., 2000; Llovera et al., 1994; Strelkov et al., 1989; Tessitore et al., 1993). The loss of muscle mass in YAH-bearing rats was associated with an increase in muscle protein degradation and activation of the ubiquitin-proteasome pathway (Baracos et al., 1995; Busquets et al., 2000; Kwak et al., 2004; Lecker et al., 2004; Llovera et al., 1994; Strelkov et al., 1989). Administration of endotoxin to tumor-bearing rodents resulted in an exaggerated cytokine response and increased mortality (Combaret et al., 2002; Grossie & Mailman, 1997; Matthys et al., 1991); however, it is not known if the hypersensitivity is extended to skeletal muscle wasting.

Hypothesis: Administration of low dose endotoxin to rats bearing an inflammatory tumor will induce a catabolic response in skeletal muscle that is greater than would be expected from tumor or endotoxin alone.

Objectives:

1. To examine the independent effects of a low dose of endotoxin and the Yoshida ascites hepatoma tumor on body weight, nitrogen balance, skeletal muscle mass, in vitro skeletal muscle protein metabolism and expression of components of the ubiquitin-proteasome pathway.
2. To determine if the effects of low dose endotoxin in the presence of the YAH tumor on the above parameters are additive or synergistic.

Chapter 4 Main Theme: Comparison between a drug-based anti-inflammatory treatment and cystine supplementation.

Potential therapies to alleviate muscle loss in cancer may include anti-inflammatory drugs or supplementation with cysteine. To determine the efficacy of each approach mice-bearing the C26 tumor were fed a control diet or the same diet supplemented with the oxidized form of cysteine, cystine. Half the

mice in each group received ibuprofen mixed in the diet. The C26 tumor is frequently used to study the role of inflammation in the development of cachexia. A progressive loss of body weight, skeletal muscle and adipose tissue during the growth of the C26 tumor occurred without a reduction in feed intake (Fujimoto-Ouchi et al., 1995; Tanaka et al., 1990). The loss of skeletal muscle protein occurs through a reduction in fractional and absolute protein synthesis rates in C26-bearing mice (Samuels et al., 2001). In addition to the reduction in muscle protein synthesis, components of the ubiquitin-proteasome and lysosomal proteolytic pathways were elevated in skeletal muscle from C26 bearing mice (Fujita et al., 1996; Kwak et al., 2004; Tilignac et al., 2002). Inflammation in the C26 model is associated with an increase in plasma IL-6, PGE_2 and acute-phase proteins (Fujimoto-Ouchi et al., 1995; Fujita et al., 1996; Matsumoto et al., 1999; Soda et al., 1995; Strassmann et al., 1992; Tanaka et al., 1989; Tanaka et al., 1990; Yasumoto et al., 1995).

Hypothesis: Chronic inflammation in the tumor bearing state is responsible for the loss of body weight and muscle mass as a direct result of increased demand for cysteine. Dietary supplementation with cystine, or treatment with an anti-inflammatory agent will reduce muscle wasting associated with tumor growth.

Objectives:

1. To compare the efficacy of ibuprofen and dietary cystine supplementation at equal tumor burden on body weight loss, skeletal muscle mass loss, muscle protein synthesis, and glutathione concentration in C26-bearing mice.
2. Determine if ibuprofen or cystine affect tumor growth and/or tumor protein synthesis.
3. Examine the effect of combination treatment with dietary cystine and ibuprofen on body weight loss, skeletal muscle mass loss, protein synthesis, glutathione concentration and tumor growth.

Chapter 5 Main Theme: Cystine supplementation during chemotherapy treatment.

Nutritional therapies that support skeletal muscle mass in cancer are often used during chemotherapy treatment. Therefore, cystine supplementation was also examined in C26-bearing mice receiving treatment with the chemotherapeutic agent, irinotecan.

Hypothesis: Cystine supplementation will promote the maintenance of body weight and skeletal muscle mass in the tumor-bearing state during chemotherapy treatment without a negative effect on the tumor response.

Objectives:

1. To determine if cystine supplementation reduces the loss of body weight and skeletal muscle mass associated with irinotecan treatment.
2. To examine the impact of supplemental cystine on the tumor response to irinotecan treatment.

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CHAPTER 2. CANCER–ASSOCIATED CACHEXIA: ALTERED METABOLISM OF PROTEIN AND AMINO ACIDS[1]

2.1 INTRODUCTION

Changes in metabolism of amino acids are not fully described for the tumor bearing state, either for animal models or for cancer patients. Several amino acids appear to show characteristic patterns of utilization in the tumor bearing state. Trials of dietary supplementation have been done more extensively for several individual amino acids in laboratory animal models, and from these we can infer the presence of possible amino acid deficiencies characteristic of the tumor bearing state; few amino acid supplementation trials have been done with cancer patients. Several minimally–invasive approaches are available to determine amino acid requirements in humans, but these have yet to be used in cancer patient populations.

2.2 AMINO ACID METABOLISM DURING PROGRESSIVE TUMOR GROWTH

Current information on protein and amino acid metabolism in cancer patients consists of a relatively sparse group of data emerging from a spectrum of animal models; human studies have employed a few highly defined patient subsets. This creates an incomplete and somewhat sketchy picture of amino acid metabolism in the tumor bearing state.

2.2.1 APPLICATIONS OF TUMOR MODELS

Cancer is an ensemble of diseases varying in biology, epidemiology and prognosis. There is no single tumor model to represent cancer, since cancer is not a single entity. Animal models for the study of cancer-associated metabolism have been the subject of several recent reviews from our laboratory (Baracos & Le Bricon, 2000; Baracos, 2001; Le Bricon et al., 2001). There is

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considerable diversity of tumor types, stages of tumor growth, and background control diets used in studies of laboratory rodents. Investigators working in this area have not yet worked together to develop a panel of tumor models that are considered representative of cancer–associated nutrient metabolism in man. The lack of a consensus set of animal models for investigations into metabolism and nutrition in cancer is an impediment to current understanding because it is impossible to reconcile results obtained in totally different models. Unfortunately, there is no single animal model for which metabolic utilization of more than one or a few amino acids has been studied. Several reports exist where the metabolism of one specific amino acid has been determined in a tumor model, but very often that tumor has not also been studied with regard to other amino acids or other aspects of metabolism.

Another important point is the majority of work has focused on the tumor bearing state *per se*, and while this formally constitutes the cancer–associated metabolic change, the clinical reality usually includes superimposed alterations in amino acid metabolism attributable to radiotherapy, chemotherapy and surgical intervention. Much less work has focused on amino acid metabolism in the treated, tumor bearing state.

Amino acid metabolism is especially complex because the utilization of at least the 20 amino compounds used for protein synthesis must be accounted for. Key alterations in amino acid metabolism may additionally include compounds not used for protein synthesis, such as ornithine, citrulline, carnosine, creatine, and taurine. Because the metabolism of amino acids is interrelated, a full description of amino acid metabolism in a tumor model would be of interest. Such a global appreciation of amino acid metabolism in animal models remains elusive.

Animal studies can illuminate which amino acids may be preferentially utilized by the host and tumor. A particular advantage of animal studies is that they permit assessment of the ability of certain amino acids to promote or inhibit

tumor growth. By contrast, quantitative aspects of amino acid metabolism determined in animal studies are very difficult to relate to cancer patients. It is especially important that consideration be given to the magnitude of tumor burden in animal studies. We have estimated that a Morris hepatoma 7777 in the rat, at 0.2% of body weight, captures 2.0% of the animal's daily nitrogen balance (Le Bricon et al., 1995a). Notably, this represents a 10-fold difference between the expected nitrogen capture by the tumor based on its mass versus its actual metabolic activity. This may be a characteristic of hepatomas, which are derived from liver cells, the key site of amino acid catabolism. As few detailed balance studies exist, the quantity of amino acid capture by other tumors is unknown. At higher tumor burdens, the tumor represents a significant nitrogen trap. For example, at 8.8% of body weight the daily nitrogen balance of the tumor is equivalent to 150% of the daily retention of nitrogen from the diet (Le Bricon et al., 1995a). Protein and amino acid metabolism in animals bearing a tumor of up to 30% of body mass have been reported, but the clinical relevance of these models is questionable, since human tumor burdens are usually in the range of 1% of body weight or less.

2.2.2 Amino acid metabolism in clinical studies

The generalization of many metabolic studies in cancer patients is affected by methodological issues such as: recruitment of patients at different points in the course of their disease and the difficulty in recruiting representative cohorts among these patients. Clinical studies on amino acid metabolism in cancer patients are relatively few, and like the animal models, usually concern a small sample from a particular patient population. For example, elevated alanine metabolism has been described in early-stage and advanced gastrointestinal cancer patients (Shaw & Wolfe, 1987) and advanced non small-cell lung cancer patients (Leij-Halfwerk et al., 2000), but it is not clear whether this represents a generality for cancer at other sites or at what time in the disease trajectory altered alanine metabolism may have become evident.

2.3 ALTERATIONS OF AMINO ACID METABOLISM IN THE TUMOR-BEARING STATE

The various observations on amino acid metabolism in the tumor bearing state remain to be fully woven into a coherent mosaic. However, recognizing the limitations stated above, it is possible to identify modifications of amino acid utilization in the tumor bearing state, in several global categories, based on available evidence.

The total and relative amounts of dietary essential amino acids required are altered. The rate and type of protein synthesis would be expected to drive total amino acid requirements. Protein synthesis, on a whole-body basis, is frequently elevated and this is one of several manifestations of a hypermetabolic state. Elevated whole-body protein turnover has been reported (Fearon et al., 1988; Jeevanandam et al., 1984; Norton et al., 1981) in tumor bearing animals and in lung, colorectal and other cancer patients relative to healthy age-matched populations or patients with non-malignant disease.

There is a change in the relative amounts of different amino acids utilized, in association with a shift from peripheral protein synthesis towards the viscera. Skeletal muscle protein mass is progressively depleted, while hepatic protein mass is maintained or may even increase. This redistribution has been known for about 30 years and was initially described in animal models (Emery et al., 1984b; Kawamura et al., 1982; Norton et al., 1981). Deficits of muscle protein synthesis have been verified in human subjects using stable isotope approaches (Emery et al., 1984a; Lundholm et al., 1976). More recently, numerous studies have demonstrated increased synthesis of hepatic secretory proteins including albumin (Fearon et al., 1998), fibrinogen (Preston et al., 1998) and C-reactive protein (Barber et al., 1999; Falconer et al., 1995; McMillan et al., 1998; Wigmore et al., 2001). Pancreatic cancer patients experiencing weight loss have also been reported to have elevated albumin synthetic rates as determined using a flooding dose technique with $^{2}H_{5}$-phenylalanine (Fearon et al., 1998). Despite an increased synthetic rate, the

cancer patients had lower plasma albumin concentrations and intra-vascular albumin mass compared to age- and height-matched healthy controls (Fearon et al., 1998). Moreover, these same pancreatic cancer patients had elevated synthesis of the acute-phase protein fibrinogen and elevated concentration of C-reactive protein, suggesting an ongoing acute-phase response (Preston et al., 1998).

Acute-phase proteins contain a higher amount of aromatic and sulphur amino acids compared to skeletal muscle protein (Reeds et al., 1994). Albumin contains a high content of cysteine and methionine (about 77 mg sulfur amino acids per g albumin versus 43 mg sulphur amino acids per g skeletal muscle protein) (Barker & Putnam, 1984; Reeds et al., 1994). When present, the acute-phase response represents a factor promoting altered amino acid requirements.

Amino acids normally considered dietary non-essential for humans become conditionally essential in the diet. Amino acids such as glutamine, arginine and cysteine may become conditionally essential in the tumor bearing state. This appears to be connected with elevated utilization of these amino acids. For example, elevated metabolic energy expenditure in the presence of anorexia creates an environment for high rates of gluconeogenesis, and the amino acids alanine and glutamine are key precursors for this process. An early paper by Shaw and Wolfe (Shaw & Wolfe, 1987) evaluated glucose and urea kinetics in healthy volunteers and in early-stage and advanced gastrointestinal cancer patients. They reported a stage-dependent elevation of gluconeogenesis and ureagenesis that were not suppressed in response to glucose infusion or total parenteral nutrition. Leij-Halfwerk et al. (2000) confirmed this result in advanced non small-cell lung cancer patients using a primed constant infusion of glucose and alanine and ^{31}P magnetic resonance spectra *in vivo* to show elevated hepatic gluconeogenic intermediates. These results illustrate a larger degree of enhancement of gluconeogenesis with more advanced disease and in patients with more intense rather than slower weight loss. Elevated metabolic rate

appears to be driven by pro-inflammatory cytokines or by excess adrenergic stimulation (Barber et al., 1999; Barber et al., 2000; Hyltander et al., 2000).

A deficit of available amino acid supplies from the diet, in the presence of increased requirements for both dietary essential and non-essential amino acids, is a primary driver of the catabolism of endogenous protein reserves in skeletal muscle. Skeletal muscle tissue dominates the body reserve of amino acids, which are mobilized under conditions where the dietary supply does not match the demand for these compounds. The major metabolic fates of mobilized muscle amino acids are gluconeogenesis and protein synthesis in extra-muscular tissues. The internal re-distribution of amino acids is not necessarily an efficient process, and there is some evidence that mobilized muscle protein is not a perfect match for whole–body amino acid requirements in every physiologic or pathologic state (Reeds et al., 1994). This has the same net effect as feeding an unbalanced amino acid mixture, with amino acids present in excess being lost to oxidation.

Amino acid mobilization may be driven by the secretion of different tumor–specific catabolic mediators. For example, a proteolysis–inducing glycoprotein of tumor origin, provokes intense protein catabolism in skeletal muscle (Tisdale, 2002; Todorov et al., 1996). In animal models, tumor–derived cytokines and eicosanoids have also been implicated in initiation of protein catabolism (Ross & Fearon, 2002). The signals for protein catabolism secreted by a tumor are not linked to a purposeful use of amino acids, and since this protein mobilization is not necessarily coordinated with host protein synthesis, oxidative losses will result.

At substantial disease burden the tumor may become a quantitatively important player in whole-body amino acid utilization. As discussed above for animal models, there is a point in tumor progression where the acquisition of amino

acids by the tumor becomes quantitatively important. This may be considered to occur only in advanced disease, however its clinical relevance is unknown.

Surgery, radiotherapy and chemotherapy are associated with large metabolic changes in substrate utilization. It is important to appreciate the metabolic change due to treatment factors. Apart from a very small number of cancers deemed untreatable or too advanced except for palliative intervention, the vast majority of cancer patients receive aggressive therapy within the limits of tolerance. Various specific amino acids may be required for healing after surgery, tissue injury in the gut and/or bone marrow after systemic therapy (Le Bricon et al., 1995b; Ziegler, 2002). However, the amino acid supply required to support such processes is not well defined.

2.4 THE METABOLISM OF SPECIFIC AMINO ACIDS IN CANCER

2.4.1 TUMOR METABOLISM OF AMINO ACIDS

Tumors, as a class, appear to have unique patterns of amino acid metabolism. This is related to their generally high proliferation rate and the use of amino compounds as biosynthetic precursors of multiple classes of molecules (**TABLE 2.1**). Protein biosynthesis is a primary determinant of tumor amino acid use, and all 20 amino acids are used in this process. Several publications have shown that the fractional synthetic rate of protein of tumors is high relative to other tissues in the tumor bearing animal (Norton et al., 1981). The fractional synthesis rate of colorectal tumors in humans was in the range of 17.2 to 33.9%/day and in breast tumors was 5.3 to 15.9%/day (Heys et al., 1991). The impact of this increased amino acid use will depend on tumor synthetic rate and mass.

Glutamine, arginine and sulphur-containing amino acids are amino acids for which elevated tumor utilization has been established for other processes in addition to protein synthesis. Tumor cells obtain a relatively high proportion of fuel for energy metabolism from complete and partial glutamine oxidation (Lazo,

1981; Le Bricon et al., 1995a). However, tumor cells have a low capacity to synthesize glutamine (Matsuno & Hirai, 1989) and instead rely on systemic glutamine from the host (Chance et al., 1988; Sauer et al., 1982). The rapid proliferation rate of tumor cells also requires enhanced production of the bioactive products of arginine metabolism, including nitric oxide and polyamines. Elevated whole-body ornithine turnover in tumor-bearing rats (Buffkin et al., 1978) supports the increase in ornithine and polyamine concentrations during growth of the tumor (Marquez et al., 1989; Seiler et al., 1985). Clinically, polyamine excretion is associated with tumor burden (Durie et al., 1977; Hyltander et al., 1998). Unlike nitric oxide production, the conversion of ornithine to polyamines results in arginine net loss. Similar to arginine, methionine is required by tumor cells to support proliferation through its essential role in methylation reactions, polyamine formation and initiation of protein synthesis. Several tumor cell lines are dependent on methionine uptake, attributed to low levels of methionine synthase activity (Kenyon et al., 2002).

Some tumors exhibit atypical amino acid use, and one example of these is the carcinoid tumors, a class of neuroendocrine tumors, which produce amines or peptides depending on the site of origin (Jensen & Norton, 1997). Serotonin is among the major bioactive substances released by active carcinoid tumors, and in carcinoid patients there is a massive diversion of tryptophan for serotonin production.

Dependence upon various amino acids by some tumor lines has led to the investigation of amino acid deprivation to reduce tumor growth. An early nutritional approach involved formulation of diets lacking amino acids essential for tumor growth. Diets deficient in arginine or methionine slowed tumor growth in animal models; anti-metabolites blocking glutamine metabolism have been investigated as chemotherapeutic agents and have been successful in reducing tumor growth in rats (Breillout et al., 1990; Chance et al., 1988). These approaches are associated with the problem of toxicity in the case of anti-

metabolites and of inducing amino acid deficiency in the host as well as the tumor (Harvie et al., 2002).

2.4.2 AMINO ACID METABOLISM IN THE TUMOR-BEARING HOST

Amino acid metabolism of the host and tumor interact. A tumor may capture amino acids, reducing availability to host tissues, and selective capture may be especially problematic in a situation when total dietary intake of amino acids is reduced. The host animal or person also responds to the presence of the tumor. Tumor-derived secretory products may alter host metabolism of amino acids in various tissues. Host response to the tumor may include endocrine and immunological changes that also impact metabolism.

The overall impact of a tumor on whole-body amino acid metabolism will inevitably depend upon the size of the tumor. More often than not, the tumor burdens used in rodent models are relatively large compared to the size of tumors in human cancer. Therefore, caution needs to be taken in interpreting and applying the findings to the clinical setting. These studies have, however, provided useful information on the pattern of amino acid metabolism in the tumor-bearing state.

2.4.2.1 GLUTAMINE

Uptake and utilization of glutamine by the tumor is a possible mechanism for low plasma glutamine levels and compromised host glutamine availability in tumor-bearing animals (Chen et al., 1991; Chen et al., 1993; Souba et al., 1988). Clinically, plasma glutamine levels are reduced in patients with cancer of the gastrointestinal tract (Bennegard et al., 1984; Kubota et al., 1992), however inadequate dietary intake often accompanies these cancers making it difficult to conclude that tumor glutamine metabolism is responsible for alterations in plasma concentrations.

Intramuscular glutamine formation and release are increased in tumor-bearing animals (Chen et al., 1991), and these are attributed to enhanced rates of muscle protein breakdown and subsequent nitrogen donation from elevated catabolism of intramuscular branched-chain amino acids. Elevated turnover of glutamine in muscle from tumor bearing rats (de Blaauw et al., 1997) is associated with a reduction in intramuscular glutamine concentration (Chen et al., 1991). Intracellular glutamine is an important regulator of muscle protein synthesis, and low concentrations may contribute to muscle loss. The liver can either produce or consume glutamine depending on the physiological state. However, the influence of tumors on hepatic glutamine metabolism is unclear. Both hepatic glutamine uptake (Easson et al., 1998; Easson et al., 2000; Fischer et al., 1997; Inoue et al., 1995a; Inoue et al., 1995b) and release (Inoue et al., 1995a) have been reported to be increased in rats bearing large tumors, without an increase in hepatic glutamine oxidation (Fischer et al., 1997). Even more unclear is the effect that tumors have on other tissues that are high consumers of glutamine, such as enterocytes and immune cells.

2.4.2.2 ARGININE

Weight losing cancer patients have lower plasma concentrations of arginine compared to well-nourished and malnourished controls in the fasted state (Bennegard et al., 1984; de Blaauw et al., 1997; Norton et al., 1985). In malnourished and well nourished patients with or without cancer, plasma arginine levels are not raised in response to feeding (Bennegard et al., 1984). Elevated rates of protein catabolism (Emery et al., 1984a; Lundholm et al., 1976) and gluconeogenesis (Leij-Halfwerk et al., 2000; Shaw & Wolfe, 1987) enhance urea production in cancer. Hepatic arginine uptake (Easson et al., 2000) and arginase activity (Marquez et al., 1989) are elevated in tumor-bearing rats indicating that there might be an increase in urea-cycle activity and arginine demand. Unlike glutamine, the capacity for the host to increase arginine biosynthesis is limited. Inadequate arginine intake is not associated with up-regulation of its synthesis (Castillo et al., 1993) and its precursor, citrulline, is

not formed at an elevated rate in tumor bearing rats (de Blaauw et al., 1997), which further limits arginine production. Also, alterations in glutamine availability and utilization in the tumor-bearing state (see section 2.4.2.1) may be a factor compromising the intestinal synthesis of citrulline.

A generalized stimulation of arginine use is associated with pro-inflammatory cytokines, which mediate the expression of inducible nitric oxide synthase (iNOS) in macrophages, other immune cells, Kupffer cells and skeletal muscle (Bedard et al., 1997; Moncada & Higgs, 1991). However, the quantitative importance to arginine utilization is unknown. Muscle nitric oxide is associated with impaired insulin-stimulated glucose uptake and oxidative stress (Bedard et al., 1997; Kapur et al., 1997), both of which are observed in cancer. The role of nitric oxide in cancer-associated muscle wasting is not known, yet the presence of a pro-inflammatory cytokine response in cancer suggests that iNOS expression and nitric oxide production are stimulated. Nitric oxide synthase inhibition prevents muscle wasting in a murine model of cachexia (Buck & Chojkier, 1996); however, this effect has not been shown clinically or in cachexia associated with cancer.

2.4.2.3 SULPHUR AMINO ACIDS AND GLUTATHIONE

Plasma cyst(e)ine (Hack et al., 1998) and glutathione (Lang et al., 2000) levels are lowered in cancer patients. As well, an increased cystine to acid soluble thiol ratio, which indicates alterations to redox status, has been associated with a loss in body cell mass in subjects with various types of advanced cancer (Hack et al., 1998). These changes are reflective of a reduction in the availability of sulphur amino acids in the tumor-bearing state attributed to tumor utilization of methionine and inadequate dietary intake. Also, the host response to tumors increases the metabolic demand for sulphur-containing amino acids for the synthesis of glutathione and acute-phase proteins (Grimble, 1990). Hepatic glutathione levels are increased in tumor-bearing mice while hepatic sulphate levels decrease, suggesting an increase in cysteine incorporation into

glutathione, resulting in reduced cysteine catabolism to sulphate within the liver (Hack et al., 1996). By contrast, intracellular glutathione is lowered in muscle from tumor-bearing mice (Hack et al., 1996), suggesting that hepatic cysteine utilization receives priority in the tumor-bearing state when cysteine availability is limited. Impaired intramuscular glutathione is associated with reduced oxidative metabolism in tumor-bearing mice, which can be reversed by supplementation with cysteine (Ushmorov et al., 1999).

2.4.2.4 ALANINE

As with glutamine, alanine release from muscle is accelerated in the tumor-bearing state (Holm et al., 1997). Protein catabolism alone does not account for the relatively large release of alanine. Increased glycolytic rate and subsequent alanine production from pyruvate enhances alanine release from muscle. Hepatic conversion of alanine provides pyruvate for gluconeogenesis through the alanine-glucose cycle as well as nitrogen for urea synthesis. Patients with advanced cancer have elevated rates of hepatic alanine conversion to glucose (Leij-Halfwerk et al., 2000) and gluconeogenesis in general (Shaw & Wolfe, 1987). Plasma alanine concentration has been reported to be increased, decreased or unchanged in cancer depending on the site of sampling, the degree of malnutrition and glycolytic rate. Alanine provision is not likely to reduce its release from muscle, however adequate non-protein energy, particularly glucose may be beneficial in slowing alanine-glucose cycling.

2.4.2.5 BRANCHED-CHAIN AMINO ACIDS

The plasma concentrations of the branched-chain amino acids (BCAA), leucine, isoleucine and valine are lowered in patients with cancer of the gastrointestinal tract (Bennegard et al., 1984; Clarke et al., 1978) and in tumor-bearing animals (Kurzer et al., 1988). Leucine in particular has a key role in the regulation of skeletal muscle protein metabolism (Nair et al., 1992). Leucine promotes muscle protein synthesis, and the metabolites of leucine catabolism inhibit muscle proteolysis (Mitch & Clark, 1984). Unlike other amino acids, the initial

step in the catabolism of leucine, isoleucine and valine takes place almost exclusively in extrahepatic tissue including skeletal muscle. Transamination of the BCAA to their respective α-keto-acid derivatives by BCAA transferase (BCAAT) in muscle supplies amino nitrogen for alanine, glutamate or glutamine synthesis, providing a non-toxic transportation of ammonia from the muscle to the liver for detoxification. Following transamination, the branched chain keto-acids can be further catabolized for fuel within the muscle itself or transported for utilization in other tissues.

Tumor-bearing rats have increased oxidation of leucine *in vivo* compared to pair-fed controls (Goodlad et al., 1981; Paxton et al., 1988). Increased activity of BCAAT and branched chain α-keto-acid dehydrogenase have been reported in tumor bearing rats (Argiles & Lopez-Soriano, 1989; Paxton et al., 1988). Enhanced leucine oxidation by skeletal muscle for use as fuel may be the result of impaired glucose and fatty acid metabolism. Insulin resistance, decreased glucose uptake by the muscle, and reduced mitochondrial transport of fatty acids in skeletal muscle impede the use of glucose and fatty acids for fuel by muscle. BCAA supplementation versus feeding at requirement increased muscle protein synthesis and improved nitrogen balance without changing tumor weight (Kawamura et al., 1985; Schaur et al., 1980), suggesting that the requirements for one or more of the BCAA is elevated in the tumor-bearing state.

2.4.2.6 Aromatic amino acids

It is generally accepted that tyrosine and phenylalanine plasma concentrations are elevated in cancer (Kokolis & Ziegler, 1977) and this likely is a reflection of increased muscle proteolysis. On the other hand, conflicting reports regarding tryptophan metabolism reflect its more complex metabolism. Studies have reported both elevated and lowered concentrations of tryptophan in a variety of cancer types (Huang et al., 2002; Naini et al., 1988). Free tryptophan may be increased in cancer, but this is most likely reflective of a decrease in the binding

of tryptophan to albumin, as total tryptophan is normal or decreased (Cascino et al., 1991; Rossi Fanelli et al., 1986). Cytokines enhance tryptophan catabolism to kynurenine (Murr et al., 2000); thus, immune stimulation in cancer may be partially responsible for the reduction in tryptophan levels. Tryptophan is the direct precursor for serotonin formation. Lowered serotonin levels have been associated with depression. In patients with colorectal cancer a reduction in serum tryptophan levels was correlated with lower quality of life (Huang et al., 2002). In contrast, elevated levels of tryptophan in the brain and subsequent serotonin synthesis may have a role in the loss of appetite common in cancer (Meguid et al., 1992; Rossi Fanelli et al., 1986).

TABLE 2.1 Tumor utilization of amino acids.

Process	Amino acids
Protein synthesis	All
ATP production	Glutamine
Nucleotide synthesis	Glutamine
Polyamine synthesis	Arginine, ornithine, methionine
Nitric oxide synthesis	Arginine
Methyl group transfer	Methionine
Serotonin synthesis	Tryptophan

2.5 MANIPULATION OF AMINO ACID SUPPLIES IN THE TUMOR-BEARING STATE

Current nutritional approaches are based on the concept of manipulating nutrient mixtures to alter the balance between the host and the tumor, in a manner that favors the host overall. Supplementation of amino acids that have become limiting for the function of the host would be expected to improve nutritional status and tolerance to treatments, and to limit morbidity and mortality. The identification and supplementation of limiting amino acids in cancer patients has potential in alleviating muscle loss, anti-oxidant status and improving immunity. It has been suggested that supplementation may pose a risk of enhancement of tumor growth. It has alternatively been suggested that appropriately formulated amino acid mixtures may interfere with tumor metabolism or have immuno-stimulatory properties, allowing more efficient anti-tumor immunity.

A factor in the use of supplementary amino acids is the administration by oral or intravenous routes. Providing amino acid supplements would be expected to have different outcomes depending on route of administration. Intravenous administration of amino acids affects the requirements due to the bypass of first-past metabolism in the gut and liver. For example, the BCAA requirement in parenterally-fed piglets is half of the requirement for piglets fed an elemental enteral diet (Elango et al., 2002). Also, bypassing the gut with intravenous feeding may compromise the de novo synthesis of dietary non-essential amino acids, such as arginine (Brunton et al., 1999) .

2.5.1 GLUTAMINE

The size, protein synthetic rate and DNA content of tumors are not affected by glutamine administration compared to tumor-bearing animals given isonitrogenous, isocaloric glutamine-free diets (Austgen et al., 1992; Kaibara et al., 1994; Yoshida et al., 1995). This is not the outcome that would have been predicted based on the observation that glutamine is required by tumor cells, suggesting that dietary influences must be evaluated in vivo. Glutamine

supplementation however, did improve whole-body nitrogen retention, increase protein synthetic rates and glutamine content in muscle and small intestine of tumor-bearing rats (Austgen et al., 1992; Kaibara et al., 1994; Yoshida et al., 1995).

2.5.2 ARGININE

Animal studies of arginine supplementation suggest that in at least some cases tumor growth is stimulated. Increased tumor growth was observed with either arginine or citrulline parenteral supplementation but not with ornithine in rats bearing the Ward colon tumor (Grossie et al., 1992; Grossie, 1996). Arginine supplementation also increased tumor protein synthetic rate in human breast cancer (Park et al., 1992) but not head and neck cancer (Caso et al., 1996). Others have found that arginine parenteral supplementation has no effect on tumor growth and that arginine supplementation improves whole-body nitrogen retention and muscle protein synthesis in tumor-bearing rats (Oka et al., 1993; Oka et al., 1994; Ye et al., 1992). Ornithine and α-ketoglutarate provide metabolic precursors for the formation of arginine and glutamine and a product for enteral nutrition containing this mixture did not stimulate growth of either the Yoshida hepatoma or Morris Hepatoma (Le Bricon et al., 1995a; Robinson et al., 1999). An amino acid mixture containing glutamine and arginine promoted deposition of lean body mass in non small cell lung cancer patients without any reported side-effects (May et al., 2002). There is not a clear explanation of why parenteral arginine would increase tumor growth in some studies and not others, but it may be related to the type of tumor.

2.5.3 CYSTEINE

A concern with cysteine supplementation is that methionine will be spared and will in turn promote tumor growth. This has not yet been investigated in animal models. Providing supplemental oral N-acetyl-cysteine for 4 weeks (400mg three times a week) was reported to improve quality of life, normalize redox state and increase plasma albumin levels and body cell mass in fifty patients

with various forms of inoperable cancer (Hack et al., 1998), suggesting that cysteine becomes conditionally essential in cancer. Importantly, the survival curve from the start of treatment to 550 days, was not different between the group that received supplementation and cancer patients who did not, which indirectly suggests that supplemental cysteine did not enhance tumor growth.

2.6 Application of new methods in the study of amino acid metabolism in cancer

Several techniques have been applied to study the metabolism of protein and amino acids in tumor-bearing animals and in clinical cancer populations including plasma amino acid profiles (Bennegard et al., 1984; Kubota et al., 1992) and protein turnover using both flooding dose and constant infusion of labeled amino acids have been used in cancer patients (Emery et al., 1984a; Fearon et al., 1988; Fearon et al., 1998; Heys et al., 1991; Preston et al., 1998) to determine in vivo protein synthesis of liver, muscle, tumors and plasma proteins. However, there are a few recently developed methodologies for identification of limiting amino acids and the quantification of amino acid requirements that have yet to be applied to cancer and will be discussed further. An important theme in the approaches used is to limit patient burden, as considerable symptoms and functional loss are associated with advanced disease.

2.6.1 Determination of amino acid requirements

Despite the evidence for altered amino acid metabolism and suggestions of beneficial effects of amino acid supplementation in cancer (May et al., 2002), amino acid requirements of cancer patients have not been determined. Recent advances in methodology include minimally invasive techniques for the determination of the requirement for specific amino acids, and these merit utilization in cancer patients.

2.6.1.1 *Indicator amino acid oxidation (IAAO)*

This approach is based on the principle that the oxidation of an indicator amino acid decreases once amino acid requirements are met (Brunton et al., 1998; Zello et al., 1995). Using this technique, breath and urine are the only samples required following the consumption of diet with a varying amount of the study amino acid and administration of the indicator amino acid tracer (Bross et al., 1998; Brunton et al., 1998). The IAAO technique has been used to determine amino acid requirements in healthy adults, neonates, children with liver transplant and in individuals with metabolic disorders (Bross et al., 1998; Brunton et al., 1998).

2.6.1.2 *The plasma amino acid response to an infusion of an amino acid mixture*

This method has been utilized to identify the amino acids that limit protein synthesis in HIV/AIDS (Laurichesse et al., 1998). This method is based on the principle that if an amino acid is limiting, its plasma concentration will not rise during an amino acid infusion, because of its use for protein synthesis. By contrast, infusion of an amino acid that is already present at or above required amounts, will result in a steep rise in its plasma concentration. A similar approach has been developed to manipulate parenteral amino acid formulation to meet the specific needs of hospitalized patients in an intensive care unit (Berard et al., 2002). The linear regression of plasma plateau concentrations of amino acids in response to an amino acid infusion was used to determine which amino acids were oversupplied or undersupplied in each individual patient (Berard et al., 2002). The administration of a parenteral amino acid formulation that corrected these imbalances was then given for 5 days and resulted in improved nitrogen balance (Berard et al., 2002). The identification of limiting amino acids in cancer is an important step in determining appropriate dietary supplements that will promote lean tissue gain, however to date this has not been accomplished in cancer patients.

2.7 SUMMARY

Current information on protein and amino acid metabolism in cancer consists of a sparse group of data, mostly from animal models. The aromatic, sulphur-containing and branched-chain amino acids are suggested to have increased utilization in the tumor-bearing state. Also, some dietary non-essential amino acids, including cysteine, arginine and glutamine, appear to become conditionally essential. Appropriately formulated amino acid mixtures can be expected to alleviate muscle loss, improve tolerance to treatments or have immune-stimulatory properties and shift the metabolic balance toward anabolism. However, lack of formal assessment of amino acid requirements in cancer patients represents a large gap in current knowledge.

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CHAPTER 3. A PRO-INFLAMMATORY TUMOR THAT ACTIVATES PROTEIN DEGRADATION SENSITIZES RATS TO CATABOLIC EFFECTS OF ENDOTOXIN[2]

3.1 INTRODUCTION

Muscle wasting in advanced cancer is associated with impaired functioning, intolerance to chemotherapy and mortality (Ross et al., 2004; Vigano et al., 2000). Numerous factors may have a role in the development of muscle wasting, but the end result is a shift in the catabolic-anabolic balance that promotes the breakdown of body protein (Baracos, 2002).

The Yoshida ascites hepatoma 130 (YAH) in rats is a tumor with rapid growth and progressive development of cachexia. YAH induces a loss of skeletal muscle mass through an enhanced rate of muscle protein degradation involving the activation of the intracellular ATP-ubiquitin-dependent proteolytic pathway (Baracos et al., 1995). This pathway involves three enzymes that ubiquinate proteins for degradation by the 26S proteasome (Tisdale, 2002). Ubiquitin activating enzyme (E1) activates and transfers ubiquitin to an ubiquitin conjugating enzyme (E2) which cooperates with an ubiquitin protein ligase (E3) to conjugate ubiquitin to the substrate. E3 is responsible for recognizing and targeting proteins for degradation. Several types of E3s have been identified, and at least three have been found to have increased expression in states of muscle atrophy, including YAH (Lecker, 2003). Of these ligases, muscle specific ring finger (MuRF-1) and atrogin-1/MAFbx have higher up-regulation in muscle atrophy, suggesting a role in disease associated muscle wasting (Lecker et al., 2004).

In tumor models, including the Yoshida hepatoma, pro-inflammatory cytokines are clearly established to be mediators of protein catabolism. Levels of tumor

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necrosis factor alpha (TNFα) are increased in physiological fluids (Tessitore et al., 1993), and a causal role in elevated catabolism is demonstrated through the use of highly specific interventions such as anti-TNFα antibodies, soluble TNFα receptors, and drugs that block TNFα production such as pentoxifylline (Combaret et al., 1999; Costelli et al., 1993; Llovera et al., 1998). There is some evidence that the TNFα response to endotoxin is exaggerated in tumor-bearing rats compared to non-tumor-bearing rats (Combaret et al., 2002). Related to this finding, studies using endotoxin have demonstrated an increase in its lethality in rats bearing the Ward colon tumor (Grossie & Mailman, 1997) and mice bearing the Lewis lung carcinoma (Matthys et al., 1991).

The presence of activated protein degradation and an enhanced sensitivity of tumor-bearing animals to pro-inflammatory signals would be expected to result in an enhanced catabolic response in tumor-bearing animals. Here we hypothesized that in the Yoshida hepatoma, bacterial endotoxin would elicit a disproportionately large activation of muscle protein catabolism, compared to non-tumor-bearing animals. To test this hypothesis we used a low dose endotoxin to determine the impact of a mild catabolic stimulus on muscle wasting, nitrogen balance and skeletal muscle protein degradation in rats bearing the Yoshida hepatoma and in non-tumor-bearing controls. Low doses of endotoxin (400 μg /kg body weight) produce a mild response that includes fever, metabolic response and cytokine receptor gene expression in muscle (Goodman, 1991; Zhang et al., 2000).

3.2 MATERIALS AND METHODS

3.2.1 STUDY DESIGN

All studies were conducted in accordance to Canadian Council on Animal Care Guidelines and were approved by the institutional Animal Policy and Welfare Committee. Male Sprague Dawley rats (n=28; Charles River, Quebec) were used as hosts for the Yoshida hepatoma. Rats were housed in individual wire metabolic cages throughout the study period. The room was temperature and humidity controlled with a 12 hour light and dark cycle. All rats consumed a diet of laboratory chow (LabDiet 5001, PMI Nutrition International, St. Louis, MO) which met all rodent nutrient requirements (National Research Council, 1995). Weight and food intake were measured daily.

Rats were randomly allocated to one of four treatment groups following 1 week adaptation to environment and diet. Treatment variables were tumor and low dose endotoxin (lipopolysaccharide, LPS). Tumor cells, maintained in liquid nitrogen, were initially implanted into non-study rats (n=2). Ascites fluid was harvested after 5 days of tumor growth and injected immediately into study rats (n=14). YAH (50μL i.p.) was implanted on day 0. After 4 days of tumor growth (day 4), treatment with saline or LPS (400 μg E coli O55:B5/kg s.c.) was given. Twenty-four hours later (day 5) rats were killed by CO_2 asphyxiation. Urine and feces were collected for the 24-hour period before and after LPS or saline treatment for determination of nitrogen (N) balance. Muscles (gastrocnemius and soleus) were dissected, weighed and frozen in liquid nitrogen. Gastrocnemius muscle was used for expression of components of the ATP-ubiquitin proteolytic pathway. Epitrochlearis muscle was dissected and incubated for determination of protein synthetic and degradation rates.

3.2.2 ANALYSIS

3.2.2.1 N BALANCE

Urine and feces were collected for two separate 24-hour periods before and after LPS/saline treatment. Feces were dried at 60°C for 48 hours and ground.

Total urine and feces collected in each period were weighed. N content of urine, feces and diet were determined using the macro-Kjeldahl method (Bradstreet, 1965). N balance was calculated by subtracting the N output (total urinary N plus total fecal N) from dietary N intake (feed intake * N content of feed). Absorbed N was calculated for each rat by subtracting fecal N content from N intake. The difference between absorbed N and urinary N was used to determine the amount of absorbed N retained and was expressed as a percent.

3.2.2.2 MUSCLE INCUBATIONS

Muscle incubations and analysis were similar to methods described previously (Baracos et al., 1995; Strelkov et al., 1989). Epitrochlearis muscle is a small thin muscle making it ideal for muscle incubations. After dissection of both epitrochlearis muscles each were placed in a tube containing 3mL of medium. All chemicals listed below are from Sigma (St. Louis, MO) unless otherwise noted. The medium was composed of: Krebs-Ringer bicarbonate buffer (119mM NaCl, 4.8mM KCl, 1.25mM $MgSO_4$, 25mM $NaHCO_3$, 1.24mM $NaHPO_4$, 1.0mM $CaCl_2$, 2mM HEPES, pH 7.4), glucose (8mM), insulin (0.01U/mL), bovine serum albumin (0.1% w/v), leucine (0.17mM), isoleucine (0.1mM) and valine (0.2mM). Muscles were initially incubated for 30 min at 37°C with 95%O_2:5%CO_2. Muscles were then transferred to another tube containing 3mL of medium with the addition of either [^{3}H]phenylalanine (0.5uCi/mL) and phenylalanine (1mM) or cycloheximide (0.5mM) followed by incubation for 2 hours under the same conditions as described above. At the end of the incubation, muscles were transferred to tubes containing 2% perchloric acid (PCA), and 0.5mL of 16% PCA was added to the media to make a 2% PCA solution. Samples were frozen at -80°C.

3.2.2.3 IN VITRO MUSCLE PROTEIN SYNTHESIS AND DEGRADATION

Muscle protein synthesis was determined using ^{3}H-phenylalanine incorporation into muscle protein. Muscles incubated in [^{3}H]phenylalanine and phenylalanine were homogenized in 3 mL 5% trichloroacetic acid (TCA) and centrifuged for 20

min. The supernatant containing the intracellular soluble fraction was removed, and after the pellet was washed three times in 5% TCA, the protein was dissolved overnight at room temperature in 1.5mL Soluene-350 (40-60% toluene, 30-40% dimethyl diakyl quaternary ammonium hydroxide and 5-10% methanol; Packard Instruments, Meriden, CT). Disintegrations per min (dpm) in the intracellular and protein fractions were measured using a beta counter (Beckman LS 5801, Beckman Instruments, Inc, Irvine, CA). Intracellular phenylalanine concentration was measured using high performance liquid chromatography (HPLC) with pre-column ortho-phthaldialdehyde (OPA) derivatization (Sedgwick et al., 1991). Intracellular specific activity (dpm·3mL^{-1}/nmol phe·3mL^{-1}) was used as the precursor pool for muscle protein synthesis. Muscle protein concentration (mg/mg muscle) was measured using LECO FP-428 Nitrogen/Protein determinator (Leco Corporation, St Joseph, MI), and protein mass (protein concentration x muscle mass) was used to calculate the specific activity of muscle protein (dpm/mg protein). Protein synthesis rate was calculated as nmol phe incorporated into protein/mg protein over the 2 hours.

Muscle protein degradation was measured using the amount of phenylalanine released from muscle when protein synthesis was blocked by cycloheximide. The phenylalanine concentration of the media was determined using HPLC and OPA derivatization. Degradation rate was expressed as nmol phenylalanine released/mg protein over the 2 hours.

3.2.2.4 ATP-UBIQUITIN DEPENDENT PROTEOLYTIC SYSTEM mRNA AND PROTEIN EXPRESSION

Levels of mRNA for components of the ATP-ubiquitin-dependent proteolytic system (ubiquitin, $E2_{14k}$, MuRF-1 and atrogin-1/MAFbx) were measured using Northern blot analysis on RNA extracted from frozen gastrocnemius muscles using the guandium isothiocynate-CsCl method. Electrophoresis of muscle RNA on 1% (w/v) agarose containing formaldehyde was followed by transfer onto nylon membranes and cross-linked to the membranes by ultraviolet light.

Membranes were hybridized with cDNA probes encoding for ubiquitin, $E2_{14k}$, MuRF-1, atrogin-1/MAFbx or 18s rRNA. Quantification was by densitometric scanning of autoradiographs or by phosphorimager analysis, and values for ubiquitin system transcripts were normalized to the 18s rRNA to correct for differences in sample loading and transfer to membrane.

Protein levels of atrogin-1/MAFbx were measured by Western blotting. Gastrocnemius muscles were homogenized using a Polytron in PBS containing 1% (w/v) NP-40 and protease inhibitors. Following centrifugation at 10,000g, protein from the soluble fraction was separated by SDS-PAGE, and transferred to nitrocellulose membranes. Membranes were incubated with the primary atrogin-1/MAFbx antibody (gift of Regeneron Pharmaceuticals Inc, Tarrytown, NY) followed by a secondary antibody for 1 hour. Phosphorimager analysis was used for quantification, and values were normalized to GAPDH to correct for differences in sample loading and transfer to membrane.

3.2.3 Statistical analysis

SAS (Version 8.2, SAS Institute, Cary NC) was used for statistical analysis. Data were expressed as mean ± SEM. Data for atrogin-1/MAFbx mRNA were not normally distributed and were log transformed. Homogeneity of variance was tested using Levene's test and multiple variances were used if group variances were not homogenous. The effects of tumor prior to the saline/LPS administration were determined using one-way ANOVA in the SAS mixed procedure (weight, intake, and N balance day 3). For the remaining analysis, treatment (tumor, LPS) and interaction effects were determined using two-way ANOVA in the SAS mixed procedure. Differences among treatment groups were identified using *t*-test. Statistical significance was considered at $p < 0.05$.

3.3 RESULTS

3.3.1 EFFECTS OF YAH

Body weight (**TABLE 3.1**) was not different between control and tumor-bearing animals at the start of the study but the growth of the ascites tumor resulted in a higher body weight at the start of day 4 ($p = 0.049$) and 5 ($p=0.005$ vs. control). However, muscle mass (**TABLE 3.2**) was reduced (gastrocnemius $p=0.002$) in the tumor-bearing group suggesting non-tumor weight loss. Body weight increased during the final 24 hours ($p=0.007$). Tumor-bearing rats had a reduction in food intake (**FIGURE 3.1**) on day 4 ($p=0.01$) and 5 ($p=0.002$).

N intake, loss and balance data are shown in **TABLE 3.3** and **FIGURE 3.2**. On day 4 YAH-bearing rats compensated for a lower total N absorbed ($p=0.005$) through a reduction in urinary N loss ($p=0.0008$) and thus maintained N balance at levels similar to controls. Despite a similar pattern on day 5 ($p=0.0009$ for N absorbed and $p=<0.0001$ for urinary N) the tumor-bearing group had a lower N balance compared to day 4 ($p=0.04$).

YAH increased muscle protein degradation ($p=0.003$) by 37% (**TABLE 3.4**). Expression of ubiquitin and $E2_{14k}$ mRNA (**FIGURE 3.3**) were elevated in the YAH group (main effect: $p = 0.003$ and 0.01 respectively). The two ubiquitin ligase (**FIGURE 3.3 AND 3.4**) measured also had higher expression in the tumor-bearing group: the atrogin-1/MAFbx ligase mRNA and protein were increased almost 5 fold (main effect $p < 0.0001$) and 1.7 fold (main effect $p= 0.0001$) respectively, and MuRF-1 mRNA was 1.5 fold higher (main effect $p = 0.01$).

3.3.2 EFFECTS OF LPS INJECTION IN NON-TUMOR-BEARING ANIMALS

Mean body and muscle weight of rats that received LPS did not differ from those that received saline in the control groups (**TABLE 3.1**). However, unlike the control group, body weight did not increase during the 24 hours after LPS administration. Despite a decrease in N intake ($p=0.04$) and loss ($p=0.01$ for urinary N and $p=0.02$ for fecal N) compared to controls, LPS alone did not affect

significantly the total N absorbed, the percent retained or N balance (**TABLE 3.3**). As well, muscle protein content (**TABLE 3.2**), degradation (**TABLE 3.4**) and expression of ATP-ubiquitin dependent system components were not affected by LPS in control rats (Ub mRNA p=0.7, E2 mRNA p=0.5, MuRF-1 mRNA p=0.4, atrogin-1/MAFbx mRNA p=0.7 and atrogin-1/MAFbx protein p=0.6) suggesting that in the healthy state the dose of LPS used does not promote a catabolic response in muscle protein.

3.3.3 SYNERGY BETWEEN YAH AND LPS

There was a significant interaction effect (p = 0.02) between tumor and LPS treatment on weight (p = 0.003) and weight gain (p = 0.007). Tumor-bearing rats receiving LPS had a lower weight (p = 0.01) and weight gain (p = 0.0007) compared to the tumor-bearing rats that received saline despite similar food intake (**TABLE 3.1 AND FIGURE 3.1**).

LPS stimulated N loss in the tumor-bearing rats as indicated by a reduction in N balance (p=0.002) in the 24 hours following LPS administration (**TABLE 3.3**). This lowering of N retention was attributed to both a decrease in N intake (p = 0.001) and total N absorbed (p= 0.001) without a change in N loss. N absorbed was similar to rats bearing YAH alone (Figure **3.2**), however there was a drastic reduction in the percent of absorbed N retained in the tumor-bearing rats given LPS (p=0.005 vs. YAH alone). Tumor-bearing rats that received LPS were the only group with N balance significantly lower than controls (p=<0.0001 vs control and p=0.003 vs. LPS).

There was a main effect of YAH on the weight (p = 0.03) and protein content (p = 0.01) of the epitrochlearis muscle (**TABLE 3.2**); however, only the tumor-bearing rats that received LPS had lower values compared to control (p = 0.03 for weight and p= 0.04 for protein content). Protein synthesis rates were not affected by either tumor implantation or LPS treatment (CON: 0.970 ± 0.070; CON + LPS: 1.084 ± 0.051; YAH: 1.111 ± 0.094; YAH + LPS: 1.008 ± 0.097

nmol phe/mg protein/2h). The major effect of the study treatments on protein turnover were on the process of protein degradation, which was strongly elevated by the tumor (+38%) and elevated another 18% by LPS treatment in the tumor-bearing group ($p = 0.03$ vs. YAH alone) (**TABLE 3.4**). However, this activation of catabolism above that induced by the tumor alone was not associated with any further increase in the mRNA expression of the elements of the ATP-ubiquitin proteolytic system studied (Ub $p=0.4$, E2 $p=0.7$, MuRF-1 $p=0.8$, atrogin-1/MAFx $p=0.9$) or protein levels of atrogin-1/MAFbx (**FIGURE 3.4**, $p=0.3$). Since LPS did not have an effect on the mRNA expression of ubiquitin, $E2_{14k}$, MuRF-1 or atrogin-1/MAFbx, nor protein level of atrogin-1/MAFbx, the data were pooled into two groups: control or YAH-bearing (**FIGURE 3.3**).

TABLE 3.1 Initial body weight and changes in body weight before (day 4) and after (day 5) LPS or saline administration in tumor-bearing (YAH) and non-tumor-bearing (CON) rats.

Mean ± SEM	CON n=7	CON+LPS N=7	YAH n=7	YAH+LPS n=7
Weight Day 0 (g)	238 ± 3	243 ± 3	243 ± 3	245 ± 4
Weight Day 4 (g) [1]	276 ± 5 [a]	283 ± 5 [a,b]	295 ± 5 [b]	286 ± 7 [a,b]
Weight Day 5 (g) [3]	283 ± 6 [a]	286 ± 4 [a]	307 ± 5 [b*]	283 ± 7 [a]
Δ [2,3]	7 ± 2 [a,b]	3 ± 2 [b]	12 ± 3 [a]	-4 ± 3 [c]

Δ equals day 5 group mean minus day 4 group mean (effect of LPS or saline treatment). Significant main effects (ANOVA, $p<0.05$): [1] Tumor effect, [2] LPS effect, [3] Interaction (Tumor*LPS). *Indicates significant difference between day 5 and day 4 mean for that treatment group (paired t-test, $p<0.05$). Within each row: groups with the same letter are not significantly different ($p<0.05$).

TABLE 3.2 Muscle weights 24 hours after LPS or saline administration in tumor-bearing (YAH) and non-tumor-bearing (CON) rats.

Mean ± SEM	CON n=7	CON+LPS n=7	YAH n=7	YAH+LPS n=7
Gastrocnemius (mg)[1]	1533 ± 28 [a]	1498 ± 48 [a]	1335 ± 20 [b]	1331 ± 50 [b]
Soleus (mg)	106.3 ± 4.8	107.1 ± 4.6	98.1 ± 5.7	95.4 ± 4.4
Epitrochlearis (mg) [1]	31.8 ± 1.2 [a]	30.8 ± 2.0 [a,b]	28.9 ± 0.8 [a,b]	27.1 ± 1.3 [b]
Protein Mass (mg) [1]	8.5 ± 0.3 [a]	8.5 ± 0.3 [a]	7.4 ± 0.5 [a,b]	7.4 ± 0.2 [b]

Significant main effects (ANOVA, $p<0.05$): [1] Tumor effect, [2] LPS effect, [3] Interaction (Tumor*LPS). Within each row: groups with the same letter are not significantly different ($p<0.05$).

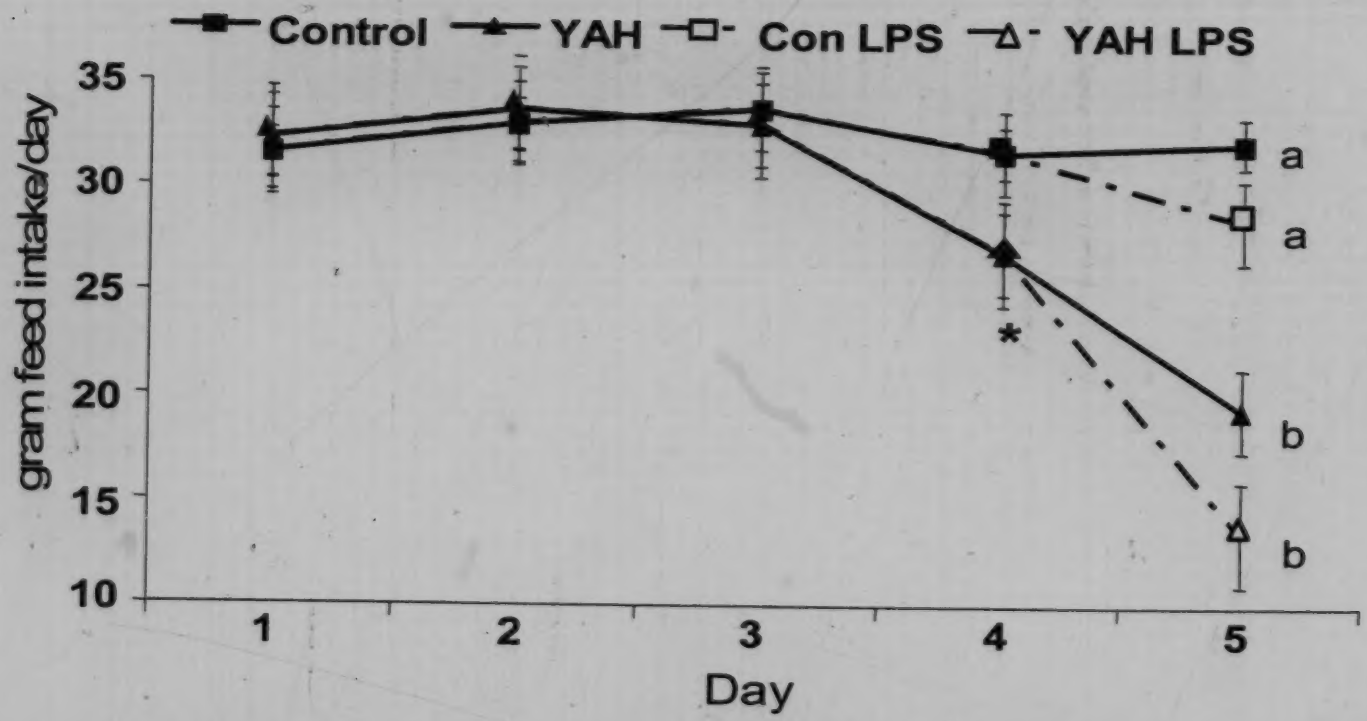

FIGURE 3.1 Daily food intake in tumor-bearing (YAH) and non-tumor-bearing (Control) rats over 5 days of treatment. Low-dose endotoxin (LPS) or saline was given at the start of day 4. n=7 rats per group. *Indicates significant YAH effect (ANOVA, $p<0.05$). Groups with the same letter are not significantly different ($p<0.05$).

TABLE 3.3 Nitrogen balance before (day 4) and after (day 5) LPS or saline administration in tumor-bearing (YAH) and non-tumor-bearing (CON) rats.

Mean ± SEM	CON n=7	CON+LPS N=7	YAH n=7	YAH+LPS N=7
		N Intake (mg/24hr)		
Day 4 [1]	1197 ± 36 [a,b]	1235 ± 60 [a]	1110 ± 30 [a,b]	964 ± 110 [b]
Day 5 [1,2]	1241 ± 66 [a]	1083 ± 91 [a]	746 ± 108 [b*]	520 ± 91 [b*]
Δ [1]	44 ± 58 [a]	-152 ± 66 [b]	-365 ± 103 [b,c]	-444 ± 79 [c]
		Urinary N (mg/24hr)		
Day 4 [1]	381 ± 13 [a]	388 ± 40 [a]	288 ± 20 [b]	251 ± 52 [b]
Day 5 [1]	462 ± 16 [a*]	397 ± 32 [a]	234 ± 17 [b]	251 ± 37 [b]
Δ [1,3]	81 ± 10 [a]	10 ± 24 [b]	-54 ± 24 [b]	0 ± 42 [a,b]
		Fecal N (mg/24hr)		
Day 4	241 ± 13 [a,b]	280 ± 21 [a]	278 ± 17 [a]	220 ± 19 [b]
Day 5 [1]	304 ± 18 [a*]	271 ± 20 [a,b]	224 ± 16 [b*]	199 ± 33 [b]
Δ [1]	63 ± 10 [a]	-9 ± 27 [b]	-54 ± 20 [b]	-21 ± 26 [b]
		N Balance (mg/24hr)		
Day 4	575 ± 21	566 ± 23	544 ± 28	493 ± 84
Day 5 [1]	475 ± 52 [a]	414 ± 85 [a]	288 ± 92 [a,b*]	70 ± 62 [b*]
Δ [1]	-100 ± 51 [a]	-152 ± 82 [a,b]	-257 ± 97 [b]	-423 ± 76 [c]

Δ equals day 4 group mean minus day 5 group mean (effect of LPS or saline treatment). Significant main effects (ANOVA, $p<0.05$): [1] Tumor effect, [2] LPS effect, [3] Interaction (Tumor*LPS). *Indicates significant difference between day 4 and 5 mean for that treatment group (paired t-test, $p<0.05$). Within each row: groups with the same letter are not significantly different ($p<0.05$).

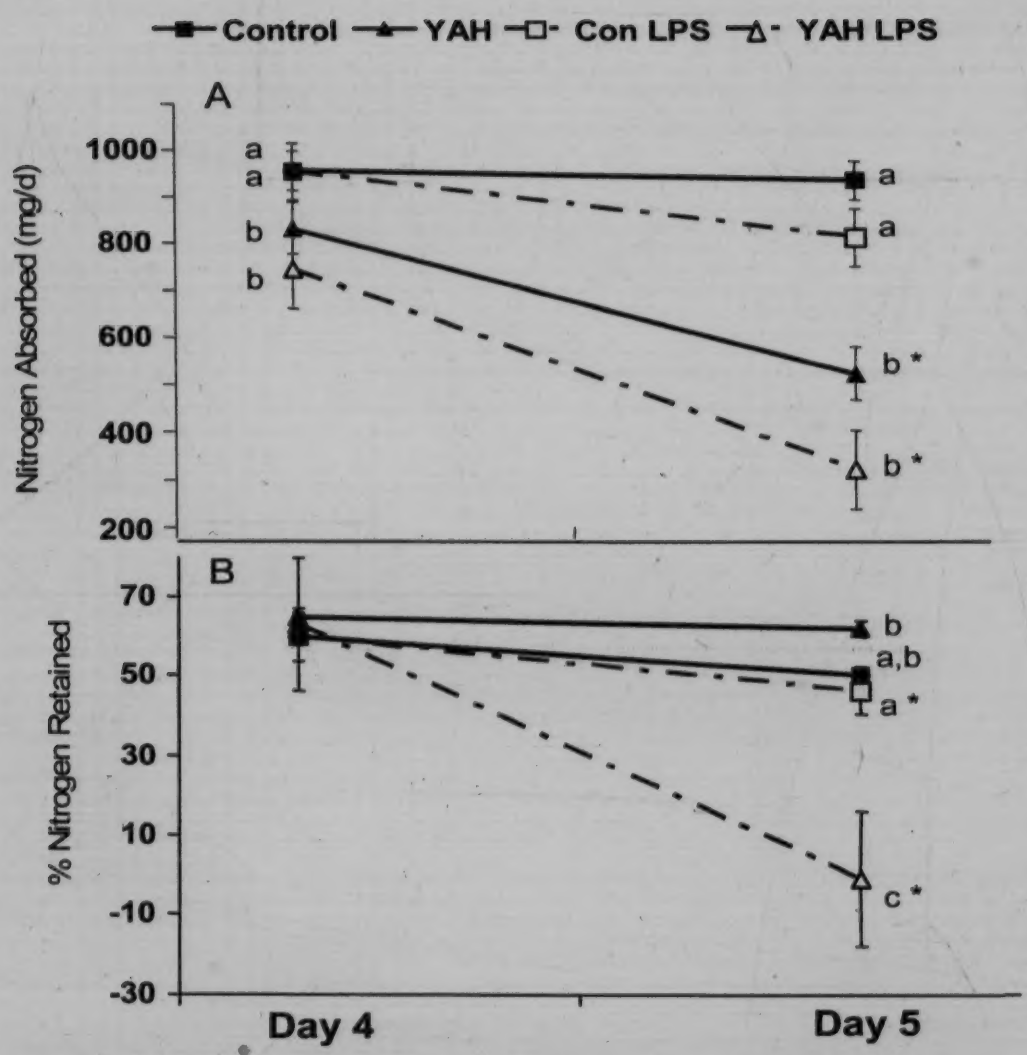

FIGURE 3.2 Nitrogen absorbed (A) and percent of absorbed nitrogen retained (B) in tumor-bearing (YAH) and non-tumor-bearing (Control) rats during the 24 hours before (day 4) and after (day 5) low dose endotoxin (LPS) or saline administration. n=7 rats per group. A: significant main effect (ANOVA, $p<0.05$) of tumor on day 4 and 5,, and B: significant main effect of LPS and an interaction effect (tumor*LPS). For each day groups with the same letter are not significantly different ($p<0.05$). *Indicates significant difference between day 4 and 5 for that group (paired t test, $p<0.05$).

TABLE 3.4 In vitro skeletal muscle protein degradation (nmol phe/mg protein/2 h) 24 hours after LPS (+LPS) or saline (-LPS) administration in YAH-bearing (+tumor) and non-tumor-bearing (-tumor) rats.

Treatment	-Tumor	+Tumor	Δ
-LPS	2.112 ± 0.147 [a]	2.906 ± 0.163 [b]	0.794 ± 0.220
			+37.6%
+LPS	2.164 ± 0.180 [a]	3.438 ± 0.182 [c]	1.274 ± 0.255
			+58.9%
Δ	0.052 ± 0.232	0.532 ± 0.244	
	+2.4% NS	+18.3%	

Δ indicates the effect of LPS or tumor. Significant main effect of tumor (ANOVA, $p<0.05$). Groups with the same letter are not significantly different ($p<0.05$). n=7 per group.

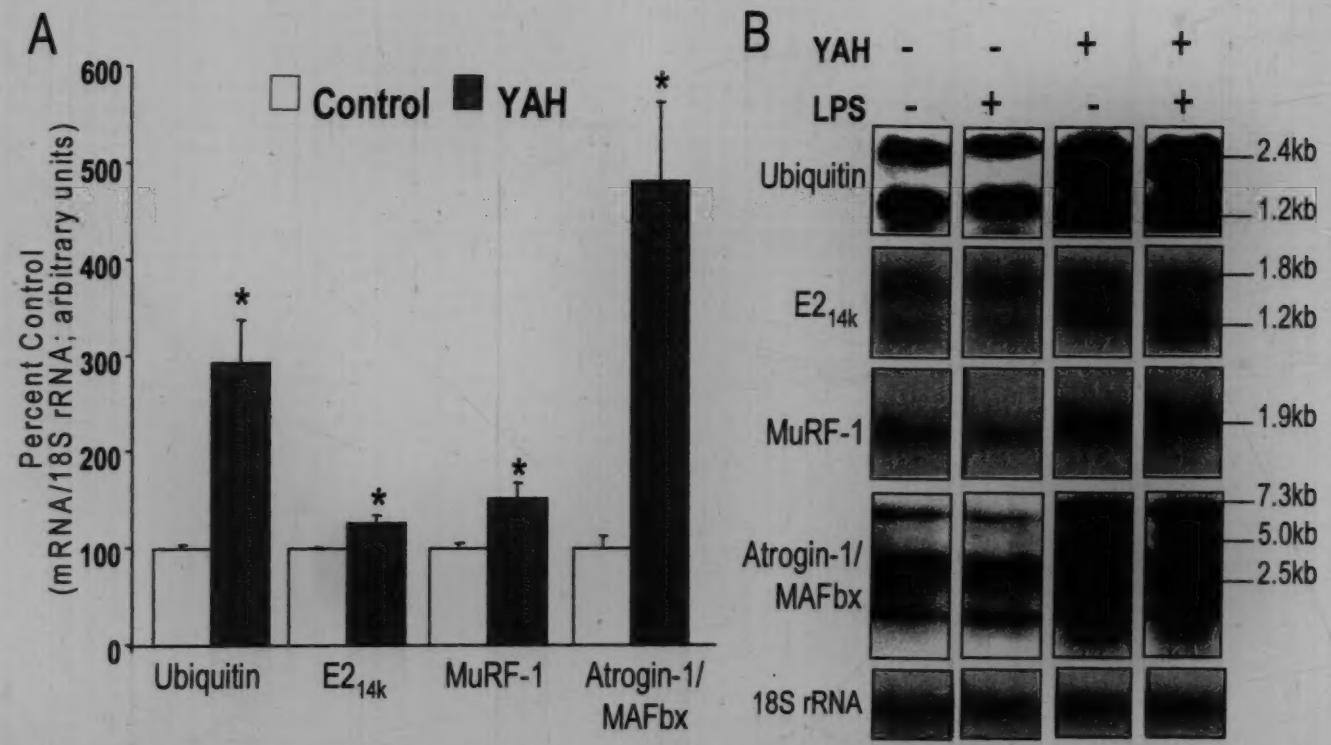

FIGURE 3.3 A. mRNA expression of components of the ATP-ubiquitin proteolytic system in the gastrocnemius muscle from tumor-bearing (YAH, n=13) and non-tumor-bearing (Control, n=14) rats 24 hours following low dose endotoxin (LPS) or saline administration. LPS had no effect in either Control or YAH rats therefore data was pooled. YAH was in the fifth day of tumor growth. Results are expressed as a percent of control. *Significant main effect of tumor (ANOVA, $p<0.05$). B. Representative Northern blots for the components of the ATP-ubiquitin proteolytic system and 18S rRNA for Control and YAH rats given LPS or saline with analyzed bands indicated. mRNA levels were standardized using 18S rRNA.

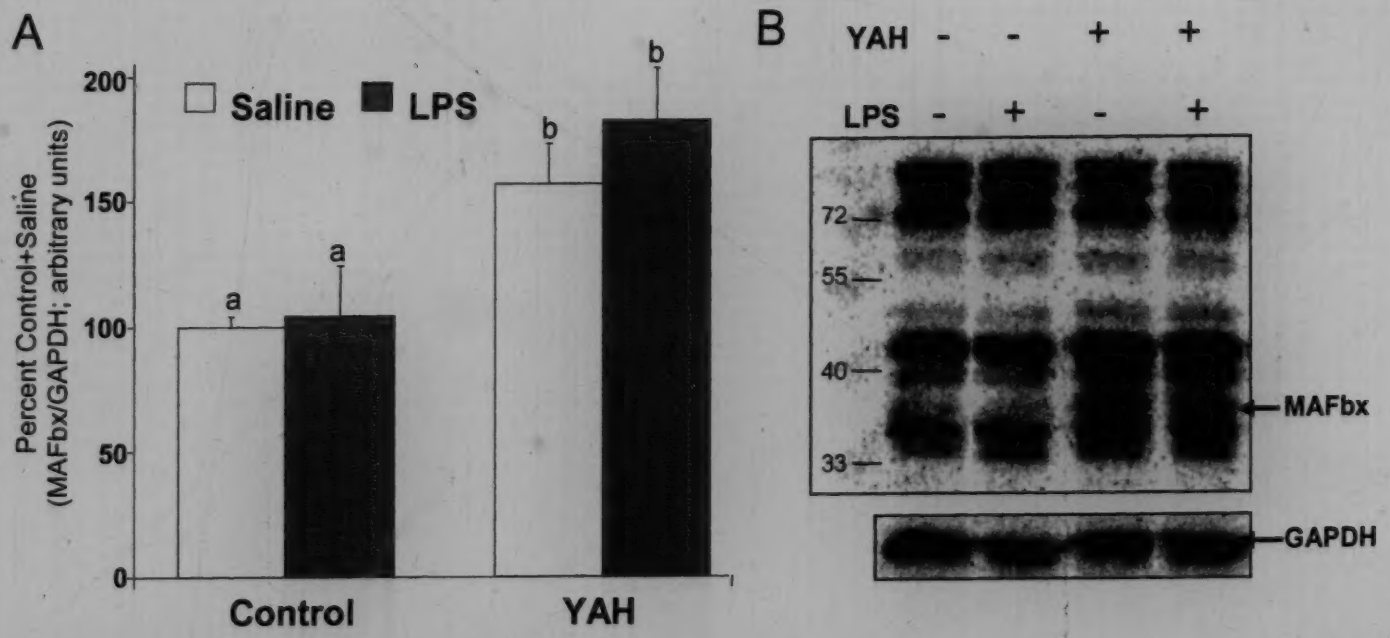

FIGURE 3.4 A. Atrogin-1/MAFbx protein in the gastrocnemius muscle from tumor-bearing (YAH) and non-tumor-bearing (Control) rats 24 hours following low dose endotoxin (LPS) or saline. YAH was in the fifth day of growth. Results are expressed as a percent control. There was a main effect of tumor (ANOVA, p=0.0001). Group size: Control n=4, Con+LPS n=7, YAH n=3, YAH+LPS n=7. Groups with the same letter are not significantly different. B. Representative Western blots for atrogin-1/MAFbx (40.9 kDa) with molecular weight markers on the left. The indicated band migrates at ~38 kDa which is consistent with it being atrogin-1/MAFbx, given the error of molecular weight estimation based on electropheretic migration of prestained standard markers. In support of this assignment, this band, like the mRNA for atrogin-1/MAFbx (Bodine et al., 2001) is only present in skeletal muscle and heart, but not in brain, kidney, lung, live, testis, and spleen (data not shown). Protein levels were standardized using GAPDH.

3.4 Discussion

The primary catabolic effects of tumors on skeletal muscle protein have been well-established in animal models (Baracos, 2000), and here we hypothesized a possible sensitization of protein catabolism to secondary factors in the tumor-bearing state. Our study shows that a superimposed inflammatory stimulus in the tumor-bearing state results in a larger catabolic response than would be expected based on the independent responses to tumor and endotoxin alone. Low-dose endotoxin reduced nitrogen retention and elevated skeletal muscle proteolysis when a tumor, known to be associated with catabolism induced by TNFα and eicosanoids, was present. The tumor model used produces an inflammatory response. Thus activation of proteolytic systems by a primary inflammatory response might predispose skeletal muscle to catabolism by a secondary stimulus such as endotoxin.

Endotoxin produces a classic response meditated by pro-inflammatory cytokines (Fink & Heard, 1990). A variety of studies have established evidence for a heightened sensitivity to endotoxin in various pathological states. Studies using a "two hit" model in rats have shown that activation of inflammatory genes by hemorrhagic shock primes macrophages for an exaggerated cytokine (TNFα) response to low dose endotoxin (Fan et al., 1998; Powers et al., 2003). This theme is seen in the literature on tumor-bearing animals. Rats bearing the Ward colon tumor had a mortality rate of 83%, compared to 8% in control rats in the 24 hours following the administration of endotoxin (5mg/kg) (Grossie & Mailman, 1997). A dose response study in mice bearing the Lewis lung carcinoma is also associated with an increased lethality of endotoxin (Matthys et al., 1991). The LD_{50} of endotoxin decreased from 700 μg in healthy mice to 60μg and 1μg in mice with a 2.7cm^3 and 6.0 cm^3 tumor, respectively (Matthys et al., 1991). Rats bearing a Yoshida sarcoma given 1 mg/kg endotoxin exhibited a plasma TNFα response 45-fold higher than in non-tumor-bearing controls (Combaret et al., 2002), and it is likely possible to reach a threshold for endotoxic shock under these conditions. Collectively these studies suggest a

sharp left shift in the endotoxin dose–toxicity relationship in the tumor-bearing state which may be related to an amplified production of TNFα. TNFα mediates endotoxin-induced shock as well as a catabolic response in skeletal muscle (Llovera et al., 1997; Mathison et al., 1988). The high levels of TNFα production in tumor-bearing animals may account for not only increased mortality but also for a shift in the catabolic response to endotoxin.

Animals with Yoshida hepatoma experienced a large increase in muscle protein catabolism associated with an induction of proteolytic gene expression. Although changes in muscle protein synthetic rates were not observed in the current study, the catabolic state of in vitro muscle incubations may have masked any alterations by tumor or endotoxin. In agreement with previous studies that have used this model (Baracos et al., 1995; Lecker et al., 2004), gene expression for key enzymes in the ubiquitin-proteasome pathway were elevated. At least four E3s have been found to have increased expression in states with muscle atrophy (Kwak et al., 2004; Lecker, 2003). These include: E3α/Ubr1, a second Ubr1 homolog E3αII, MuRF-1 (muscle specific ring finger) and atrogin-1/MAFbx (also known as SCF atrogin1). Studies in gene knockout mice, and differential gene analysis in rat models of atrophy, indicate that atrogin-1/MAFbx and MuRF-1 are involved in the enhanced protein degradation associated with muscle wasting (Bodine et al., 2001; Lecker et al., 2004). Mice with atrogin-1/MAFbx gene deletion demonstrate a 56% reduction in denervation-induced muscle wasting whereas the reduction in MuRF-1 gene deficient mice was 36% (Bodine et al., 2001). Atrogin-1/MAFbx mRNA expression displayed the greatest degree of YAH-induced elevation along with an increase in protein levels in our study. Despite the higher rates of skeletal muscle protein breakdown, endotoxin in YAH-bearing rats did not increase the expression of genes involved in ubiquitin conjugation, suggesting that other mechanisms of increasing substrate flux through the pathway are involved. The relative contribution of different ligases to muscle wasting associated with tumors or endotoxin is not known. Two muscle specific ligases, MuRF-1 and

atrogin-1/MAFbx, were examined in this study, and the protein level was only determined for only atrogin-1/MAFbx. Therefore it is possible that other ligases were more responsive to endotoxin in the tumor-bearing rats. E3αII mRNA expression is elevated in response to YAH, as well as TNFα (Kwak et al., 2004), and thus may have a role in sensitizing skeletal muscle of tumor-bearing rats to endotoxin. It is also possible that the increased expression of genes in the conjugation pathway is part of an increase in the infrastructure within proteolytic pathways that allows for more catabolism to take place upon the arrival of a second stimulus, endotoxin in this case. The second stimulus may, for example, cause enhanced gene translation, post-translational activation of enzymatic activity, or proteasome regulation which are sites downstream of gene transcription that may affect proteolytic rates. Also, the availability of substrates for ubiquitin conjugation may be elevated by endotoxin.

There is some evidence of a synergistic effect of catabolic factors on skeletal muscle protein breakdown. A low dose of cortisol, which had a minimal effect on muscle protein catabolism in normally active healthy young men, elevated skeletal muscle protein breakdown rates threefold in the same subjects after bed rest for 2 weeks (Ferrando et al., 1999). Although both inactivity and cortisol stimulate muscle protein breakdown, co-administration results in muscle protein breakdown greater than expected from the sum of both. Endotoxin at relatively high doses (10 mg/kg) in rats has been shown to increase skeletal muscle breakdown through activation of the ATP-ubiquitin proteolytic pathway (Chai et al., 2003; Combaret et al., 1999). In the design of the current study, we selected a much lower dose of endotoxin, which would be expected to cause little or no perturbation in protein metabolism. Accordingly, the effects on N metabolism elicited by low dose endotoxin in healthy control rats were minimal; however like the study of muscle sensitivity to cortisol, muscles of tumor-bearing rats had an enhanced degradative response to endotoxin. Our related work with the same low dose of endotoxin which demonstrated an induction of TNF and IL-6 receptors in skeletal muscle (Zhang et al., 2000) provides a

possible basis for this sensitization. This pattern of enhanced sensitivity of muscle protein degradation to catabolic factors may be an important component of complex wasting disorders where tumor, inflammation or infection, inactivity, elevated levels of glucocorticoids and other factors may frequently be simultaneously present. Our study highlights the complexity involved in defining the mechanism responsible for, and treatment of, muscle wasting in disease states. The etiology of wasting may not be attributable to a single factor, but rather to a series of factors that disastrously potentiate each other's catabolic effects, and the magnitude of the overall effect can not be predicted based on the separate effects of each.

The unfortunate outcomes of tumor and endotoxin/inflammation may be a feature of tumors which are themselves associated with some degree of inflammatory mediator production or host inflammatory response. The Yoshida hepatoma used here and the other tumor models where enhanced sensitivity to endotoxin was demonstrated (Combaret et al., 2002; Matthys et al., 1991) are associated with an inflammatory response. Many human tumors that are associated with wasting of skeletal muscle are associated with indices of inflammation (Fearon et al., 1988; Fearon et al., 1991; Staal-van den Brekel et al., 1997), and these in turn are known to have prognostic significance and to be related to shortened survival (Falconer et al., 1995; Scott et al., 2002). The current study relates clinically to the metabolic response to infections in cancer patients. Infections are very common in patients with cancer (Nagy-Agren & Haley, 2002) and are often the cause of death in advanced cancer patients (Homsi et al., 2000; Inagaki et al., 1974). Our results generate the speculation that episodes of infection or inflammation may be associated with exaggerated catabolic responses in the tumor-bearing state.

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CHAPTER 4. IBUPROFEN, BUT NOT SUPPLEMENTAL CYSTINE, IMPROVES SKELETAL MUSCLE MASS IN A TUMOR MODEL OF CHRONIC INFLAMMATION

4.1 INTRODUCTION

Inflammation and muscle wasting are common features in several conditions, including acute trauma, sepsis, cancer, HIV/AIDS, congestive heart failure, chronic obstructive pulmonary disease, and inflammatory bowel disease (Delano & Moldawer, 2006). The loss of skeletal muscle is of particular importance because muscle wasting is associated with increased mortality, physical disability, and impaired immune response, poor wound repair (Demling, 2007; Janssen et al., 2004; Kotler et al., 1989; Simons et al., 1999). Cytokines mediate inflammation and directly act on skeletal muscle through activation of protein catabolism (Argiles et al., 2005). Cytokines also stimulate the synthesis of proteins and other compounds involved in inflammation which can reduce amino acid availability for synthesis of muscle protein (Obled et al., 2002; Reeds et al., 1994).

Cysteine is an amino acid that is required in relatively high amounts during an inflammatory response for the production of acute-phase proteins and the anti-oxidant glutathione (Grimble, 1990; Malmezat et al., 2000). The addition of cysteine to an amino acid mixture increased skeletal muscle mass and improved nitrogen balance in a rat model of acute inflammation induced by a bacterial injection (Breuille et al., 2006). Plasma cyst(e)ine and glutathione levels are lowered during inflammatory conditions, suggesting insufficient cysteine availability for glutathione synthesis (Droge & Holm, 1997). The provision of N-acetylcysteine or a high-cyst(e)ine protein in patients with cancer increased body weight and lean body mass (Hack et al., 1998; Tozer et al., 2008). However, the effectiveness of dietary cyst(e)ine supplementation in the prevention of skeletal muscle loss associated with inflammation has not been established in cancer.

We hypothesized that chronic inflammation associated with the tumor-bearing state contributes to body weight loss and muscle wasting through an increase in cysteine demand. This hypothesis would predict that treatment with an anti-inflammatory agent or dietary cystine supplementation would mitigate the muscle wasting associated with tumor growth. Combination treatment with a nutrient and a drug that have different molecular targets constitutes a new strategy (Rasmusen et al., 2007). We examined the effect of ibuprofen, dietary cystine supplementation, and the combination of the two treatments on muscle wasting, protein synthesis, sulphur amino acids and glutathione in mice bearing an inflammatory tumor, the colon 26 (C26) adenocarcinoma. The C26 tumor causes progressive wasting and an inflammatory response mediated by IL-6 (Fujimoto-Ouchi et al., 1995; Soda et al., 1995; Tanaka et al., 1990; Zhou et al., 2003).

4.2 MATERIALS AND METHODS

4.2.1 STUDY DESIGN AND ANIMALS

Male BALB/c mice (3 weeks old) were obtained from Charles River (St. Constant, QC) and housed in wire top shoebox cages (1-4 mice per cage) in a temperature and humidity controlled room on a 12-hour light-dark cycle. Following 3 weeks adaptation, each cage was randomized into one of the following groups: non-tumor-bearing reference fed the control diet (Ref n=20); C26-bearing fed the control diet (C26 n=21); C26-bearing fed the cystine supplemented diet (C26+Cys n=21); C26-bearing fed the control diet containing ibuprofen (C26+Ibu n=19); or C26-bearing fed the cystine supplemented diet containing ibuprofen (C26+Cys+Ibu n=21). Body weight and tumor volume were measured every 2 days and food intake was measured daily. Tumor-bearing mice were killed at equal tumor burden (~5% body weight). The full study was completed in three blocks, each block containing mice from all treatment groups. The study was conducted in accordance to Canadian Council on Animal Care Guidelines and was approved by the institutional Animal Policy and Welfare Committee.

4.2.2 COLON 26 TUMOR

Mice in C26-bearing groups received 1.5×10^6 tumor cells subcutaneously on day 0. Reference mice received a subcutaneous injection of saline. The length, width and depth of the tumor was measured using a micro-caliper and the formula used to calculate tumor volume (mm^3) was: (a*b*c)/2 where a is tumor length, b is tumor width and c is tumor depth.

4.2.3 DIET

The AIN-76 based semi-purified casein diets (**TABLE 4.1**) were isocaloric, isonitrogenous and contained the same amount of total methionine (free methionine plus methionine from casein). The diet met or exceeded nutrient requirements for mice (National Research Council, 1995). The diet had a high fat content (20% w/w) with a polyunsaturated:saturated fatty acid ratio of 0.4

and a n-6:n-3 fatty acid ratio of 18 which is similar to the fat composition of a typical North American diet. The amount and composition of fat in our study diets was expected to facilitate tumor growth and inflammation (Birt, 1997; Robinson et al., 2002; Simopoulos, 2006). All mice received the control diet during the adaptation period, and the cystine supplemented diet was started on day 0. Cysteine is a strong reducing agent with the ability to bind minerals (Baker & Czarnecki-Maulden, 1987); therefore, we supplemented with the oxidized form of cysteine, cystine. The addition of either cysteine or cystine to a diet with minimal methionine supported normal growth in mice (Friedman & Gumbmann, 1984). The diets were designed to contain 6% of protein as cyst(e)ine compared to 0.4% found in the control diet. A cyst(e)ine content of 4.7-6% of total amino acids has been suggested as the requirement in disease states (Obled et al., 2002). Additional casein was used to make the control diet isonitrogenous (Chambon-Savanóvitch et al., 1999).

4.2.4 ANTI-INFLAMMATORY TREATMENT

Half the C26-bearing mice in each dietary group were treated daily with the non-specific cyclooxygenase (COX) inhibitor, ibuprofen, to suppress the inflammatory response. The dose of ibuprofen used has been demonstrated to attenuate inflammation in mice (Heneka et al., 2005). Ibuprofen was mixed into the diet (0.4 mg ibuprofen/g diet) to provide a dose of 60mg/kg/d. COX inhibitors in mice bearing the C26 tumor increased body weight and muscle mass (Davis et al., 2004; Hitt et al., 2005; McCarthy et al., 2004; Tanaka et al., 1989). However, since tumor growth was suppressed by the COX inhibitor, it is not clear if the improvements in body weight and skeletal muscle mass were independent of effects of a lower tumor burden. To account for the effect of different growth rates we studied the mice at equal tumor burden (~5% of body weight).

4.2.5 *FLOODING DOSE*

Protein synthesis in skeletal muscle (gastrocnemius), liver and tumor were measured using the flooding dose technique as described previously (Garlick et al., 1980; Samuels et al., 2001). Mice were given an intraperitoneal injection of 1.5 µmol phenylalanine and 0.9 µCi [^{3}H]-phenylalanine/g body weight, and sacrificed by CO_2 asphyxiation 15 minutes later. Blood was obtained by cardiac puncture followed by dissection of gastrocnemius muscle, liver, tumor and spleen. Tissues were weighed and frozen in liquid nitrogen. Blood was centrifuged (1,500 x g for 15 min at 4°C) to separate plasma. Plasma and tissue samples were stored at -80°C until analysis.

4.2.6 *LABORATORY ANALYSIS*

4.2.6.1 PROTEIN SYNTHESIS

Specific activity of the supernatant and hydrosylate fractions of each tissue was determined using the conversion of phenylalanine to β-phenylethylamine followed by organic extraction (McAllister et al., 1995). The concentration β-phenylethylamine was determined by o-phthaldialdehyde (OPA) derivatization reverse phase HPLC as described previously (McAllister et al., 1995). β-Phenylethylamine standards were prepared at three levels of concentration, and each level was analyzed in duplicate or triplicate during each run. Analysis of samples demonstrated that other amino acids were not present in the extracted organic layer. Disintegrations per minute (DPM) was measured in 0.5mL of extracted samples using a beta counter (Beckman LS 5801, Beckman Instruments, Inc, Irvine, CA). The specific activities of the intracellular free (SA_f=DPM_f/nmol β-phenylethylamine$_f$) and protein bound (SA_b=DPM_b/nmol β-phenylethylamine$_b$) fractions were used to calculate protein synthetic rates using the formula: %/day = (SA_b x 100)/(SA_f x t) where t is the time between injection of flooding dose and tissue dissection in days.

4.2.6.2 *Tissue and plasma amino acid concentration*

Tissue and plasma were analyzed for methionine and taurine using reverse phase HPLC and OPA derivatives (Sedgwick et al., 1991). Concentration of total cyst(e)ine and homocyst(e)ine in tissue (oxidized+reduced) and plasma (oxidized+reduced+protein bound) were analyzed using a thiol specific HPLC method (Araki & Sako, 1987; Gilfix et al., 1997; Kuhn et al., 2000). All samples were analyzed in duplicate and calibration was done every 10 samples.

4.2.6.3 *Tissue glutathione*

Tissue (15-20mg) from muscle, liver and tumor were analyzed for total and oxidized glutathione concentration using an enzymatic assay kit (Oxford Biomedical Research, Oxford, MI).

4.2.6.4 *Plasma IL-6*

The primary pro-inflammatory cytokine in C26 tumor models, IL-6, was measured in plasma by enzyme linked immunoassay (ELISA) using murine specific kit. The BD OptEIA™ kit (BD Biosciences, Mississauga, ON) contained plates pre-coated with a monoclonal antibody specific for mouse IL-6, lyophilized recombinant mouse IL-6 standards, and reagents. The optical density was read at 450nm (Spectra Max 190 Molecular Devices Corp., Sunnydale, CA).

4.2.7 *Statistical analysis*

All statistical analyses were performed using SAS Version 8.2 (SAS Institute Inc, Cary, NC). Data were expressed as mean ± SEM. Data were transformed if not normally distributed. All analyses were completed in the mixed procedure using the Kenward-Roger method for degrees of freedom. A p value of < 0.05 was considered significant.

Reference mice were compared to the C26-bearing mice fed the control diet to determine the effect of tumor using one-way analysis of variance with the

model: $y = \mu + group_i + cage_{j(i)} + block_k + \varepsilon_{ijk}$, where μ is the overall mean, $group_i$ is the fixed effect (reference or C26), $cage_{j(i)}$ is the random effect associated with cage group, $block_k$ is the random effect associated with block (1, 2 or 3), and ε_{ijk} is the random error. Significance between reference mice and C26-bearing control mice is indicated in all tables and figures with an asterisk.

To determine the independent effects and interaction of cystine and ibuprofen treatment, tumor-bearing groups were compared to each other using a two-way analysis of variance with the factorial model: $y = \mu + diet_i + ibuprofen_j + (diet*ibuprofen)_{ij} + cage_{k(ij)} + block_l + \varepsilon_{ijkl}$, where μ is the overall mean, $diet_i$ is the diet group (control or cystine supplemented), $ibuprofen_j$ is the ibuprofen group (none or ibuprofen treatment), $(diet*ibuprofen)_{ij}$ is the interaction, $cage_{k(ij)}$ and $block_l$ are the random effects, and ε_{ijkl} is the random error. Significant main effects of cysteine, ibuprofen and the interaction between the two are noted in tables and figures with uppercase letters. Multiple group comparison among the C26-bearing groups was performed using the least significant difference method to compute p-values. Significance between the C26-bearing groups is indicated in all tables and figures with superscript lowercase letters, groups with different letters are significantly different from each other.

Repeated measures analysis with compound symmetry structure was used for daily body weight and tumor volume using the same models as above with the addition of period (day) and initial body weight for the body weight analysis.

TABLE 4.1 Diet composition

Ingredient[A] (g/kg)	Control	Cystine
High-Nitrogen Casein	250	240
L-Methionine	2.5	2.8
L-Cystine	0	13.2
Cornstarch	217.6	214.1
Dextrose	210	210
Cellulose	50	50
Vitamin Mix	10	10
Mineral Mix	50.9	50.9
Choline	2.75	2.75
Inositol	6.25	6.25
Sterine Flakes	121.4	121.4
Sunflower Oil	74.6	74.6
Flaxseed Oil	4	4
Energy (kJ ME/kg)	18912	18912
Nitrogen (g/kg)	34	34

[A]Sources for ingredients: High nitrogen casein, cornstartch, dextrose, cellulose, vitamin and mineral mix, choline, and inositol from MP Biomedical (Solon, OH); L-methionine and L-cystine from Sigma-Aldrich (Oakville, ON); soyabean sterine flakes from CanAmera Foods (Oakville, ON); sunflower oil from Canada Safeway Limited (Calgary, AB); and flaxseed oil from Holista Health, Inc. (Coquitlam, BC).

4.3 RESULTS

4.3.1 TUMOR GROWTH

Tumor growth was slower with ibuprofen treatment (**FIGURE 4.1**, overall main effect: p=0.0002) starting on day 22 of tumor growth. An interaction between cystine and ibuprofen was present (p=0.02) such that tumor volume was lowered by ibuprofen only in the control fed mice (C26 vs. C26+Ibu: p<0.0001). Therefore, the average length of tumor growth was 2 days longer for C26+Ibu group in order to achieve equal tumor burden, but the number of days of tumor growth was not significantly different between the groups (Ref: 32 ± 1 days; C26: 31 ± 1 days; C26+Cys: 30 ± 1 days; C26+Ibu: 33 ± 1 days; C26+Cys+Ibu: 31 ± 1 days). Tumor protein fractional synthesis rate was not affected by cystine, ibuprofen or combination treatment (C26: 29 ± 2 %/d; C26+Cys: 36 ± 4 %/d; C26+Ibu: 37 ± 3 %/d; C26+Cys+Ibu: 39 ± 3 %/d).

4.3.2 BODY WEIGHT

Initial body weight was not different among groups (Ref: 20.9 ± 0.2 g; C26: 21.0 ± 0.3 g; C26+Cys: 21.0 ± 0.2 g; C26+Ibu: 20.7 ± 0.3 g; C26+Cys+Ibu: 21.0 ± 0.2 g). The C26 tumor reduced body weight (**FIGURE 4.2**) compared to reference mice (overall effect: p=0.002) starting 11 days before the final day. Cystine lowered body weight compared to control fed C26-bearing mice starting 10 days before the final day (overall main effect: p=0.004) while ibuprofen increased body weight on the last two days (main effect: p=0.0003). An interaction between cystine and ibuprofen was present as indicated using repeated measure analysis (p=0.008) where ibuprofen increased body weight only in the control diet fed group (overall effect C26 vs. C26+Ibu: p=0.01), and cystine lowered body weight only in the ibuprofen treated group (overall effect C26+Ibu vs. C26+Cys+Ibu: p=0.0002).

The C26 tumor reduced total non-tumor weight gain (**TABLE 4.2**) during the study period (C26 vs. Ref: p=<0.0001). Non-tumor weight gain was lowered by cystine (main effect: p=0.01) and increased by ibuprofen (main effect: p=0.02).

However, ibuprofen increased non-tumor weight gain only in the control fed group (C26 vs. C26+Ibu: p=0.003) and cystine reduced non-tumor weight gain only in the ibuprofen treated group (C26+Cys+Ibu vs. C26+Ibu: p=0.001). The significant differences remained the same when non-tumor weight gain was expressed as grams/day, indicating that the differences with ibuprofen treatment were not due to the longer study period.

4.3.3 Food intake

Average daily food intake based on body weight was not affected by the C26 tumor, cystine, ibuprofen or the combined treatment (Ref: 166 ± 8 g/kg; C26: 157 ± 7 g/kg; C26+Cys: 163 ± 6 g/kg; C26+Ibu: 163 ± 5 g/kg; C26+Ibu+Cys: 166 ± 4 g/kg). Average daily cystine intake in the supplemented groups (2.3 ± 0.7 g/kg) was higher (main effect: p<0.0001) than in the non-supplemented groups (0.1 ± 0.0 g/kg). The average daily ibuprofen intake was 66 ± 1 mg/kg for the two groups treated with ibuprofen.

4.3.4 Plasma IL-6

The C26 tumor increased plasma IL-6 **(Table 4.3)** by over 100 fold (C26 vs. Ref: p=0.001). Cystine supplementation had no effect on plasma IL-6, whereas ibuprofen treatment reduced plasma IL-6 levels (main effect: p<0.0001).

4.3.5 Liver and spleen

Liver mass relative to non-tumor body weight was increased by the C26 tumor (**Figure 4.3**, C26 vs. Ref: p = 0.002). Ibuprofen reduced liver mass relative to body weight (main effect: p=0.004); however the effect of ibuprofen was limited to the cystine supplemented group in multiple group comparisons (C26+Cys vs. C26+Cys+Ibu: p=0.01). Fractional synthesis rate of liver protein was not affected by the C26 tumor, cystine, ibuprofen or the combined treatment (overall mean: 123 ± 5 %/d).

Spleen mass relative to non-tumor body weight was increased 4 fold by the C26 tumor (**FIGURE 4.3,** C26 vs. Ref: $p<0.0001$). Cystine and ibuprofen both reduced relative spleen mass (main effect: $p=0.0011$ for cystine and $p<0.0001$ for ibuprofen). The effect of cystine and ibuprofen was cumulative with the combination of cystine and ibuprofen resulting in the lowest relative spleen mass (C26+Cys+Ibu: vs. C26 $p<0.0001$; vs. C26+Cys $p<0.0001$, vs. C26+Ibu $p=0.001$).

4.3.6 GASTROCNEMIUS MUSCLE

Gastrocnemius muscle mass was reduced by the C26 tumor (C26 vs. Ref: $p=0.02$) but when expressed relative to body weight, there was no difference (**FIGURE 4.4**). Protein synthesis in the gastrocnemius muscle was reduced 43% by the C26 tumor (C26 vs. Ref: $p<0.0001$). Cystine did not have an impact on gastrocnemius muscle mass or percent body weight, but cystine reduced muscle protein synthesis (main effect: $p=0.04$). Ibuprofen treatment partially restored gastrocnemius muscle mass (main effect: $p=0.03$) and protein synthesis (main effect: $p=0.03$). However, cystine lowered muscle protein synthesis only with ibuprofen treatment (C26+Cys+Ibu vs. C26+Ibu: $p=0.04$), and ibuprofen partially restored muscle mass and protein synthesis only in the control fed group (muscle mass: C26 vs. C26+Ibu: $p=0.02$; muscle protein synthesis: C26 vs. C26+Ibu: $p=0.03$).

4.3.7 SULPHUR AMINO ACIDS

Plasma cyst(e)ine was reduced 27% by the C26 tumor (C26 vs. Ref: $p=0.02$) while liver cyst(e)ine concentration was increased by 46% (C26 vs. Ref: $p=0.01$), and muscle concentration was unaffected (**TABLE 4.4**). A significant interaction ($p=0.02$) between cystine and ibuprofen on plasma cyst(e)ine was present. Ibuprofen treatment increased plasma cyst(e)ine only in the control diet fed group (C26 vs. C26+Ibu: $p=0.006$). Liver cyst(e)ine concentration was lowered by ibuprofen treatment (main effect: $p=0.048$). While cystine

supplementation did not affect cyst(e)ine concentration in plasma, liver or muscle, tumor cyst(e)ine concentration was increased (main effect: p=0.009).

The C26 tumor reduced plasma methionine by 50% (C26 vs. Ref: p=0.005) but did not affect concentration in liver and muscle. Cystine, ibuprofen nor the combination altered methionine concentration in plasma, liver, muscle or tumor. Homocyst(e)ine concentration in plasma (overall mean: 14 ± 1 nmol/mL), liver (overall mean: 42 ± 3 nmol/g) and tumor (overall mean: 10 ± 3 nmol/g) was not affected by cystine, ibuprofen or the combined treatment. Taurine concentration in plasma (overall mean: 468 ± 25 nmol/mL), liver (9.3 ± 0.2 nmol/mg) and muscle (3.7 ± 0.1 nmol/mg) were not affected by tumor, cystine or ibuprofen. Ibuprofen increased (main effect: p=0.008) tumor taurine concentration (nmol/mg tumor tissue: C26: 5.1 ± 0.1; C26+Cys: 4.8 ± 0.2; C26+Ibu: 5.4 ± 0.1; C26+Cys+Ibu: 5.7 ± 0.2).

4.3.8 GLUTATHIONE

Total glutathione concentration in liver (C26 vs. Ref: p=0.01) and muscle (C26 vs. Ref: p=0.009) were decreased by the C26 tumor (**TABLE 4.5**). The lower total glutathione in liver was attributed to a lower concentration of both reduced (C26 vs. Ref: p=0.02) and oxidized (C26 vs. Ref: p=0.04) glutathione. Oxidized glutathione was below detectable levels (10 nmol/g) in half of the muscle samples analyzed; therefore, only total glutathione levels are reported for muscle. Plasma total glutathione was not affected by the C26 tumor.

There was a significant interaction between cystine and ibuprofen on total (p=0.01) and reduced (p=0.008) glutathione concentration in liver. Ibuprofen increased liver total and reduced glutathione in the control fed mice (C26+Ibu vs. C26: p=0.01 and p=0.007 respectively) which was lowered by the addition of cystine. Ibuprofen also increased muscle total glutathione (main effect: p=0.04); however, in multiple group comparisons, muscle total glutathione was increased only in the control fed mice (C26+Ibu vs. C26: p=0.04 and p=0.04 respectively).

Tumor total, reduced and oxidized glutathione concentration was not affected by cystine, ibuprofen or the combination. The ratio of tumor reduced to oxidized glutathione in liver and tumor tissue was not affected by tumor, cystine or ibuprofen.

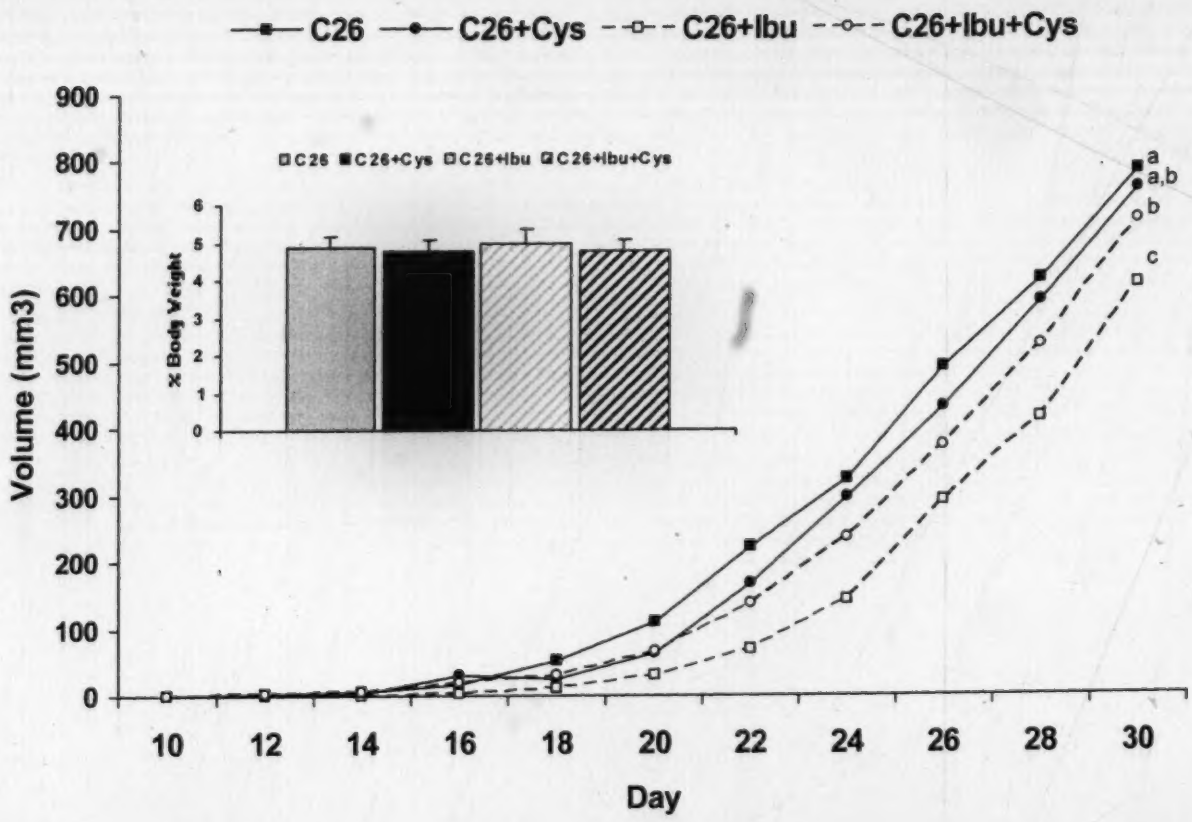

FIGURE 4.1 Tumor volume in colon 26(C26)-bearing mice fed either a control or cystine (Cys) supplemented diet with and without ibuprofen (Ibu). Inset is tumor burden at time of tissue sampling. n=19-21 per group. C26-bearing groups were compared using factorial ANOVA repeated measures; overall significance indicated by different letters. Significant main effect of ibu and interaction (cys x ibu).

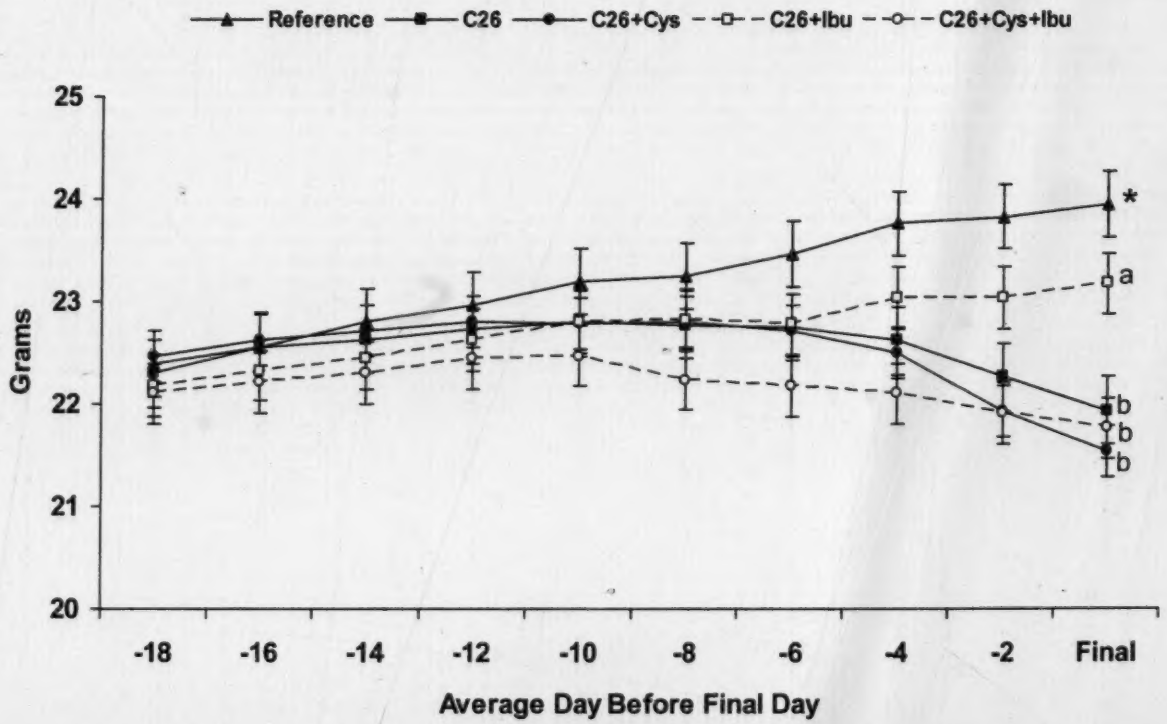

FIGURE 4.2 Body weight during the final 19 days in reference and colon 26(C26)-bearing mice fed either a control or cystine (Cys) supplemented diet with and without ibuprofen (Ibu). n=19-21 per group. Reference group was compared to C26 using ANOVA repeated measures; overall significance indicated by asterisk (*). C26-bearing groups were compared using factorial ANOVA repeated measures; overall significance indicated by different letters. Significant main effect of cys and interaction (cys x ibu) on body weight changes during the study period.

TABLE 4.2 Weight gain in reference and colon 26(C26)-bearing mice fed either a control or cystine (Cys) supplemented diet with and without ibuprofen (Ibu).

Mean ± SEM	Reference	C26	C26+Cys	C26+Ibu	C26+Cys+Ibu
Non-Tumor Total Weight Gain (g) [AB]	3.07 ± 0.13*	-0.11 ± 0.01[a]	-0.43 ± 0.40[a]	1.28 ± 0.27[b]	-0.22 ± 0.30[a]
Non-Tumor Weight Gain per Day (g/d) [AB]	0.10 ± 0.00*	0.00 ± 0.01[a]	-0.01 ± 0.01[a]	0.04 ± 0.01[b]	-0.01 ± 0.01[a]

Non-tumor total weight gain = total weight gain during study – tumor weight. Non-tumor weight gain per day = non-tumor total weight gain/number of days of tumor growth. n=19-21 per group. Reference group was compared to C26 using ANOVA; significance indicated by asterisk (*). C26-bearing groups were compared using factorial ANOVA; significance indicated by uppercase letters for main effects (A: cys, B: ibu, C: interaction) and by different lowercase letters for multiple group comparison.

TABLE 4.3 Plasma interleukin-6 (IL-6) in reference and colon 26(C26)-bearing mice fed either a control or cystine (Cys) supplemented diet with and without ibuprofen (Ibu).

Mean ± SEM	Reference	C26	C26+Cys	C26+Ibu	C26+Cys+Ibu
Plasma IL-6 (pg/mL) [B]	7 ± 3*	997 ± 270[a]	873 ± 201[a]	323 ± 156[b]	48 ± 11[b]

n=10-11 per group. Reference group was compared to C26 using ANOVA; significance indicated by asterisk (*). C26-bearing groups were compared using factorial ANOVA; significance indicated by uppercase letters for main effects (A: cys, B: ibu, C: interaction) and by different lowercase letters for multiple group comparison.

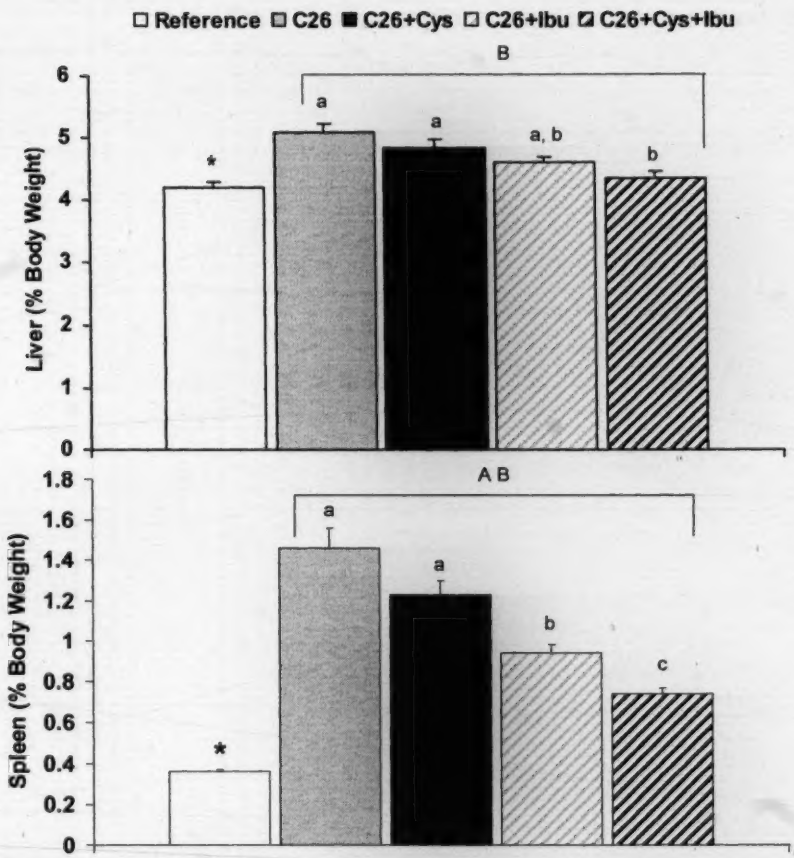

FIGURE 4.3 Relative liver (top panel) and spleen mass (bottom panel) in reference and colon 26(C26)-bearing mice fed either a control or cystine (Cys) supplemented diet with and without ibuprofen (Ibu). n=19-21 per group. Reference group was compared to C26 using ANOVA; significance indicated by asterisk (*). C26-bearing groups were compared using factorial ANOVA; significance indicated by uppercase letters for main effects (A: cys, B: ibu, C: interaction) and by different lowercase letters for multiple group comparisons.

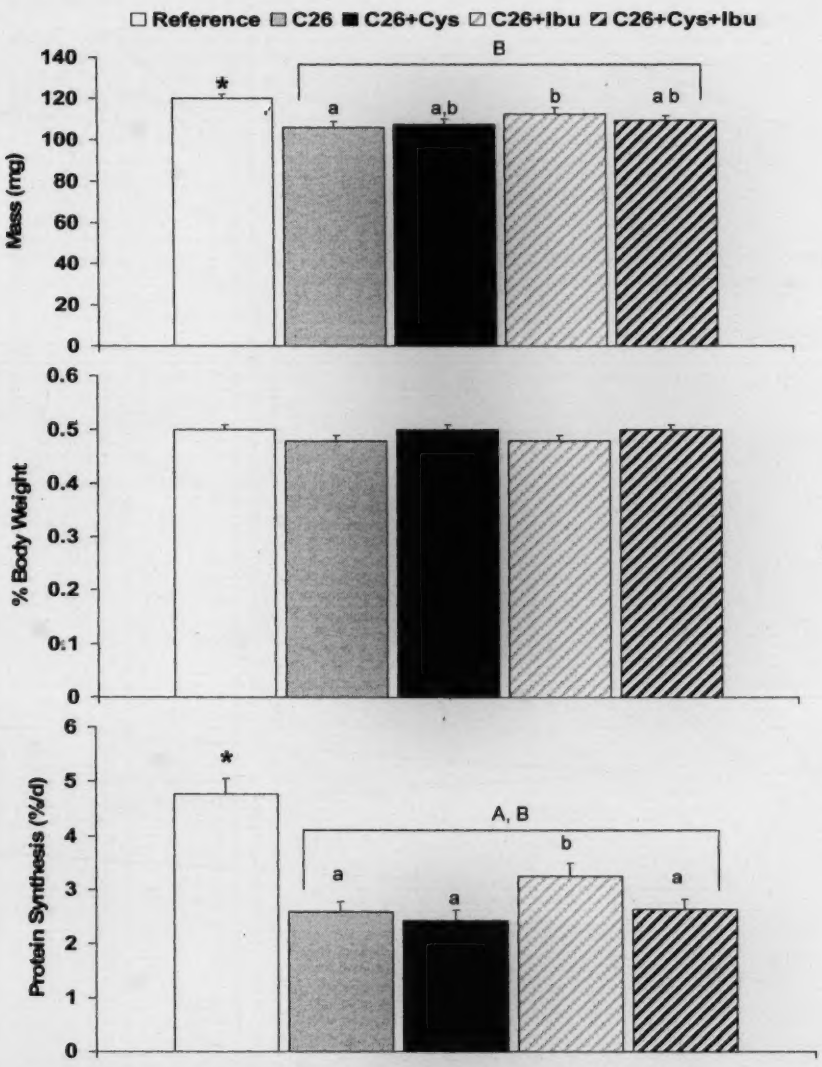

FIGURE 4.4 Gastrocnemius mass, percent of body weight and protein synthesis in reference and colon 26(C26)-bearing mice fed either a control or cystine (Cys) supplemented diet with and without ibuprofen (Ibu). n=19-21 per group. Reference group was compared to C26 using ANOVA; significance indicated by asterisk (*). C26-bearing groups were compared using factorial ANOVA; significance indicated by uppercase letters for main effects (A: cys, B: ibu, C: interaction) and by different lowercase letters for multiple group comparisons.

TABLE 4.4 Cyst(e)ine and methionine concentration in plasma, liver, muscle and tumor from reference and colon 26(C26)-bearing mice fed either a control or cystine supplemented diet with and without ibuprofen (Ibu).

Mean ± SEM	Reference	C26	C26+ Cys	C26+ Ibu	C26+Cys+ Ibu
			Plasma (nmol/ml)		
Cyst(e)ine[C]	250 ± 33*	182 ± 14[a]	199 ± 13[a,b]	228 ± 22[b]	194 ± 19[a]
Methionine	62 ± 8*	31 ± 2	40 ± 6	32 ± 5	42 ± 5
			Liver (nmol/g tissue)		
Cyst(e)ine[B]	153 ± 22*	224 ± 16	219 ± 24	207 ± 33	197 ± 18
Methionine	83 ± 14	124 ± 19	122 ± 16	114 ± 24	139 ± 25
			Muscle (nmol/g tissue)		
Cyst(e)ine	23 ± 3	34 ± 2	39 ± 6	31 ± 2	34 ± 6
Methionine	105 ± 10	75 ± 7	88 ± 9	81 ± 10	73 ± 7
			Tumor (nmol/g tissue)		
Cyst(e)ine[A]		382 ± 30[a]	465 ± 48[a,b]	429 ± 62[a]	505 ± 48[b]
Methionine		354 ± 55	332 ± 35	354 ± 25	435 ± 57

n=5-10 per group. Reference group was compared to C26 using ANOVA; significance indicated by asterisk (*). C26-bearing groups were compared using factorial ANOVA; significance indicated by uppercase letters for main effects (A: cys, B: ibu, C: interaction) and by different lowercase letters for multiple group comparisons.

TABLE 4.5 Glutathione in plasma, liver, muscle and tumor from reference and colon 26(C26)-bearing mice fed either a control or cystine (Cys) supplemented diet with and without ibuprofen (Ibu).

Mean±SEM	Reference	C26	C26+ Cys	C26+ Ibu	C26+ Cys+Ibu
			Plasma (nmol/ml)		
Total	112 ± 9	122 ± 10	115 ± 11	114 ± 8	109 ± 9
Reduced	nd	nd	nd	nd	nd
Oxidized	nd	nd	nd	nd	nd
R:O	nd	nd	nd	nd	nd
			Liver (umol/g tissue)		
Total[C]	12.9 ± 0.4*	8.4 ± 0.3[a]	9.8 ± 0.7[ab]	11.0 ± 0.9[b]	8.6 ± 0.3[a]
Reduced[C]	11.2 ± 0.3*	7.7 ± 0.6[a]	8.9 ± 0.6[ab]	10.2 ± 0.9[b]	7.8 ± 0.3[a]
Oxidized	0.8 ± 0.2*	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
R:O	17 ± 4	36 ±15	33 ±12	29 ±5	24 ± 6
			Muscle (nmol/g tissue)		
Total[B]	1588 ± 69*	1006 ± 91[a]	1125 ± 96[ab]	1263 ± 64[b]	1232 ± 70[ab]
Reduced	nd	nd	nd	nd	nd
Oxidized	nd	nd	nd	nd	nd
R:O	nd	nd	nd	nd	nd
			Tumor (nmol/g tissue)		
Total		2916 ± 200	3231 ± 468	2403 ± 462	2517 ± 335
Reduced		2683 ± 217	3106 ± 476	2166 ± 500	2436 ± 320
Oxidized		116 ± 39	62 ± 18	118 ± 27	41 ± 15
R:O		47 ± 18	76 ± 23	21 ± 9	90 ± 42

nd: not determined; R:O: reduced to oxidized ratio. n=5-7 per group. Reference group was compared to C26 using ANOVA; significance indicated by asterisk (*). C26-bearing groups were compared using factorial ANOVA; significance indicated by uppercase letters for main effects (A: cys, B: ibu, C: interaction) and by different lowercase letters for multiple group comparisons.

4.4 Discussion

This study compared the efficacy of drug-based COX inhibition to cystine supplementation in the treatment of body weight and skeletal muscle mass loss associated with the C26 tumor in mice. The effectiveness of ibuprofen on the partial restoration of skeletal muscle mass demonstrates that inflammation contributes to the development of cachexia in the tumor-bearing state. Conversely, the conjecture that dietary cystine supplementation would support maintenance of skeletal muscle mass during inflammation associated with tumor growth was not supported. Furthermore, supplemental cystine counteracted most of the positive effects of ibuprofen.

The C26 tumor reduced plasma cyst(e)ine and tissue glutathione levels. A similar reduction in muscle and liver total glutathione with lowered plasma cysteine was found in mice-bearing the methylcholanthrene (MCA)-induced tumor (Hack et al., 1996). The mechanism of lowered glutathione levels in the tumor-bearing models has not been established; however, an increase in hepatic γ-glutamylcysteine-synthetase activity in the MCA tumor model (Hack et al., 1996) suggests that hepatic glutathione synthesis was not impaired. Similar to a model of acute inflammation (Breuille et al., 2006), hepatic cyst(e)ine concentration was elevated with the C26 tumor and suggests a shift in cyst(e)ine supply to the liver or a reduction in hepatic cyst(e)ine catabolism. Tumor concentration of cyst(e)ine was about two times as high as liver concentration. Tumor cyst(e)ine uptake would induce an additional demand for cysteine in the C26 model, however, measurements for cyst(e)ine flux would be required to quantify tumor uptake of cyst(e)ine.

The alterations to cyst(e)ine and glutathione levels with the C26 tumor justified our rationale to enrich the diet with cystine. However, supplementation with cystine did not alter the reduction in weight gain, skeletal muscle mass, protein synthesis or glutathione levels in the C26 model. Cyst(e)ine intake in the current study (2.3g/kg/d) was higher than that used in a study of cysteine

supplementation in a rodent model of acute inflammation (1.0g/kg/d) that improved skeletal muscle mass and nitrogen balance (Breuille et al., 2006). Despite the high cystine provision in this study, the concentration of cyst(e)ine was not increased in plasma, liver or skeletal muscle. A difference between our study and the study in a rodent model of acute inflammation was that our diet was supplemented with cystine as opposed to cysteine. Although supplemental cystine has been established as effective as cysteine in supporting growth in young animals fed minimal dietary methionine but inadequate dietary cyst(e)ine (Baker, 2006; Friedman & Gumbmann, 1984), the relative efficacy of cystine and cysteine has not been examined in pathological states where there may be an increased demand for cyst(e)ine.

The only effect of cystine supplementation was an elevation in tumor cyst(e)ine concentration. The sulphur amino acids are a specific interest in stimulation of tumor growth due to the role of methionine in methylation reactions required for cell proliferation and the antioxidant properties of cyst(e)ine that could protect tumor cells from apoptosis (Cellarier et al., 2003; Zeisel, 2004). Cystine supplementation did not alter tumor growth, protein synthesis, methionine or glutathione levels in tumor tissue, suggesting that the higher cyst(e)ine level was not advantageous for tumor proliferation. The increase in tumor cyst(e)ine content with supplementation accounted for a very small portion of total cyst(e)ine intake. The primary utilization of dietary cystine may have been during first-pass metabolism. Activity of hepatic cysteine diooxygenase, the enzyme responsible for the irreversible first step in cysteine catabolism, is increased when dietary cysteine intake is elevated (Stipanuk et al., 2002). Therefore, oxidation of dietary cyst(e)ine during first-pass metabolism may have limited the release of cysteine into systemic circulation. The contribution of first pass metabolism to cyst(e)ine oxidation was demonstrated in rats administered cysteine by two routes; 70% of orally administered cysteine was oxidized, whereas only 40% of the intraperitoneal injected cysteine was oxidized (Stipanuk & Rotter, 1984). Skeletal muscle glutathione levels were increased in

mice bearing the MCA tumor with intraperitoneal injections of cysteine (Hack et al., 1996), suggesting that systemic administration of cyst(e)ine may be more effective at elevating cysteine availability for peripheral tissues.

COX inhibitors, including ibuprofen, have been shown previously to increase weight gain and muscle mass in the C26 model (Davis et al., 2004; McCarthy et al., 2004; Tanaka et al., 1989; Yao et al., 2005). Also, *in vitro* skeletal muscle protein synthesis was increased by a COX inhibitor in rats-bearing the Yoshida hepatoma (Strelkov et al., 1989). However, COX inhibitors also reduced tumor growth in animal models and the effects attributed to reduced inflammation could also be due to a smaller tumor burden. We examined the effects of ibuprofen on skeletal muscle when tumor mass was equal to that of tumor-bearing mice not treated with ibuprofen. Therefore, the effects of ibuprofen treatment on body weight and skeletal muscle are attributed to the reduction in inflammation independent of the effects of ibuprofen on tumor burden. Ibuprofen treatment also improved plasma cyst(e)ine and tissue glutathione levels, suggesting that the alterations to cyst(e)ine in the C26 model were due to the metabolic response to inflammation.

The ibuprofen treated group had a longer study period to overcome the limitation of comparing two groups with unequal tumor burden. On average, the ibuprofen treated group had a study period two days longer than the other groups. It is not clear if this short period of time would have had an impact on the outcome measures. When weight gain was expressed as grams per day, the ibuprofen treated group still had a higher rate weight gain, indicating that the higher weight gain with ibuprofen treatment was not due to the longer study period. However, we can not discount the possibility that the longer length of study period had an effect.

Combination treatment with macronutrients and drugs is a relatively new approach. A study that examined arginine and a statin drug in the prevention of

artheroma formation in hypercholesterolemia rabbits found that the combination of the two treatments was more beneficial than either one alone (Rasmusen et al, 2007). However, in our study the positive benefits of ibuprofen on body weight, skeletal muscle mass and the suppression of tumor growth were not present in mice treated with both ibuprofen and cystine. The mechanism of this interaction is not apparent. Although plasma IL-6 was lowered by ibuprofen, other inflammatory mediators were not measured and inflammation at the tissue level was not assessed. The anti-inflammatory action of ibuprofen, such as downregulation nuclear factor kappa B activation, would be countered if dietary cystine increased the level of other inflammatory mediators, such as tumor tumor necrosis factorα, in tissue.

Our study demonstrated the effectiveness of ibuprofen on partially restoring the loss of body weight and skeletal muscle mass associated with the C26 tumor. Treatment with COX inhibitors in patients with cancer has been shown to increase body weight (MacDonald, 2007); however, the skeletal muscle-specific effects of COX inhibitors had not been previously demonstrated independent of tumor burden. The second major finding in our study was that dietary cystine supplementation countered the effectiveness of ibuprofen. Although the association of nutrients and drugs has the potential to be beneficial, there are also risks with combination therpy. The interaction of ibuprofen with cystine underscores the importance of pre-clinical assessment of combination therapy.

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CHAPTER 5. DIETARY CYSTINE SUPPLEMENTATION IMPROVES TUMOR RESPONSE TO IRINOTECAN TREATMENT IN MICE BEARING THE COLON 26 TUMOR

5.1 INTRODUCTION

The loss of body weight and skeletal muscle mass is common in cancer and is associated with poor chemotherapy response and shortened survival (Andreyev et al., 1998; Dewys et al., 1980; Hauser et al., 2006; Ross et al., 2004). Metabolic alterations associated with weight loss in cancer include an increase in skeletal muscle protein degradation and a reduction in skeletal muscle protein synthesis (Dworzak et al., 1998; Emery et al., 1984; Fearon et al., 1988; Jagoe et al., 2002; Lundholm et al., 1976; Melville et al., 1990; Williams et al., 1999). Despite alterations in protein metabolism, the dietary amino acids required to support the maintenance of skeletal muscle mass in patients with cancer are not known. Without adequate dietary provision of the amino acids required, a reduction in amino acid availability would limit muscle protein synthesis and contribute to the loss of skeletal muscle mass in cancer.

Supplementation with a cysteine precursor, N-acetyl-cysteine or high cysteine protein increased body cell mass in patients with advanced cancer (Hack et al., 1998; Tozer et al., 2008). However, the conjecture that cysteine improves skeletal muscle mass in subjects with cancer has not been tested. Since the use of nutritional supplementation in patients with cancer will almost always be in the context of anti-cancer therapy, we examined the efficacy of cystine supplementation on body weight and skeletal muscle mass in colon 26(C26)-bearing mice that received irinotecan (Camptosar®, CPT-11) treatment. CPT-11 is topoisomerase I inhibitor used in first line therapy for recurrent or metastatic colorectal cancer. As with many chemotherapeutic agents, the cytotoxicity of CPT-11 is associated with the generation of reactive oxygen species (ROS) (Conklin, 2004). Supplemental cyst(e)ine can increase the production of glutathione, an intracellular antioxidant, which could potentially interfere with efficacy of CPT-11. Therefore, the effect of cystine

supplementation on the tumor response to CPT-11 treatment was also determined.

5.2 MATERIALS AND METHODS

5.2.1 STUDY DESIGN AND ANIMALS

Male BALB/c mice (n=36, 3 weeks old) were obtained from Charles River (St. Constant, Quebec) and housed in wire top shoebox cages (3 mice per cage) in a temperature and humidity controlled room on a 12 hour light-dark cycle. Following 3 weeks adaptation, all mice were implanted with the C26 tumor on day 0 (**FIGURE 5.1**). On day 15 four cages (n=12 mice) began to receive the cystine supplemented diet. The cystine supplemented mice (C26+CPT-11+Cys) and four cages (n=12 mice) of the control fed mice (C26+CPT-11) began daily irinotecan treatment on day 23 for 3 consecutive days. The remaining four cages (n=12 mice) were used as C26-bearing controls (C26). Body weight and food intake were measured every 2 days from day 0 to 22 and every day thereafter. Tumor volume was measured on day 23, 25 and 27. The study was completed in 4 blocks, with each block containing mice from the 3 treatment groups. The study was conducted in accordance with Canadian Council on Animal Care Guidelines, and was approved by the institutional Animal Policy and Welfare Committee.

5.2.2 COLON 26 TUMOR

The C26 tumor in mice produces progressive development of cachexia, characterized by weight loss, lipid depletion and skeletal muscle wasting (Fujita et al., 1996; Tanaka et al., 1990). All mice received 1.5 x 10^6 tumor cells through a subcutaneous injection on day 0. The length, width and depth of the tumor was measured using a micro-caliper and the formula used to calculate tumor volume (mm^3) was: (a*b*c)/2 where a is tumor length, b is tumor width and c is tumor depth.

5.2.3 DIET

The AIN-76 based semi-purified casein diets were isocaloric, isonitrogenous and contained the same amount of methionine (**CHAPTER 4, TABLE 4.1**). The diet met or exceeded nutrient requirements for mice (National Research Council,

1995). The fat content of the diet supplied 40% of energy and had a low polyunsaturated:saturated level (0.4) and high n-6:n-3 (18:1). All mice received the control diet during the adaptation period and the first 15 days of tumor growth. The cystine supplemented diet contained 6% of total amino acids as cystine, which is similar to the cyst(e)ine content of the whey protein supplement used in cancer patients (Tozer et al., 2008). This was compared to the control diet that contained 0.4% of total amino acids as cyst(e)ine. Additional casein protein was used to make the control diet isonitrogenous (Chambon-Savanovitch et al., 1999).

5.2.4 CHEMOTHERAPY TREATMENT

On day 23 the cystine supplemented mice and four cages of the control fed mice began chemotherapy treatment with CPT-11 (Pfizer Canada, Montreal, QC) for 3 consecutive days. A total dose of 300mg CPT-11/kg (100mg/kg/d x 3 days i.p.) was given to one cage of both the control and cystine fed mice (n=3 per group). This dose resulted in one death in the control diet group; therefore the dose was reduced for the remaining sets. The remaining CPT-11 treated mice (n=9 per group) were given a total dose of 240mg/kg (100mg/kg/d x 2 days plus 40mg/kg/d x 1 day i.p.). Four cages of control fed mice (n=12) served as non-treated controls and received sterile saline (100uL/d x 3 days i.p.) instead of CPT-11. Some side-effects of CPT-11 were managed with anti-cholinergic and anti-biotic prophylactic treatment. Mice were given the anti-cholinergic, atropine (2mg/kg, s.c.) 15 minutes before each CPT-11 injection to manage early-onset gastrointestinal discomfort and diarrhea. Mice were given ciprofloxacin (~10mg/kg/d p.o.) starting day 15 to prevent systemic infections associated with opportunistic pathogens. The ciprofloxacin treatment was administered in sterile drinking water (40mg/L). The control mice were also administered ciprofloxacin and atropine.

5.2.5 FLOODING DOSE

Protein synthesis in skeletal muscle (gastrocnemius) was measured using the flooding dose technique described previously (Garlick et al., 1980; Samuels et

al., 2001). Mice were given an i.p. injection of 1.5μmol phenylalanine and 0.9μCi [^{3}H]-phenylalanine/g body weight and sacrificed by CO_2 asphyxiation 15 minutes later. Blood was obtained by cardiac puncture followed by dissection of gastrocnemius muscle, liver, tumor and spleen. Tissues were weighed and frozen in liquid nitrogen. Blood was centrifuged (1,500 *g* for 15mins at 4°C) to separate plasma. Plasma and tissue samples were stored at -80°C until analysis.

5.2.6 LABORATORY ANALYSIS

5.2.6.1 PROTEIN SYNTHESIS

Specific activity of the supernatant and hydrosylate fractions of each tissue was determined using the conversion of phenylalanine to β-phenylethylamine (McAllister et al., 1995). The concentration β-phenylethylamine was determined by o-phthaldialdehyde (OPA) derivatization reverse phase HPLC as described previously (McAllister et al., 1995). β-Phenylethylamine standards were prepared at three levels of concentration, and each level was analyzed in duplicate or triplicate during each run. Analysis of samples demonstrated that other amino acids were not present after extraction. Disintegrations per minute (DPM) were measured in 0.5mL of extracted samples using a beta counter (Beckman LS 5801, Beckman Instruments, Inc, Irvine, CA). Muscle protein synthesis was measured in n=6 mice per group, and the subset groups had equal tumor burden to examine the effect of cystine supplementation independent of tumor size.

5.2.6.2 TUMOR CYST(E)INE AND GLUTATHIONE

Concentration of cyst(e)ine (oxidized+reduced) was analyzed using a thiol specific HPLC method (Araki & Sako, 1987; Gilfix et al., 1997; Kuhn et al., 2000). All samples were analyzed in duplicate, and calibration was done every 10 samples. Total and oxidized glutathione concentration were analyzed using an enzymatic assay kit (Oxford Biomedical Research, Oxford, MI).

5.2.7 STATISTICAL ANALYSIS

Statistical analysis was performed using SAS Version 8.2 (SAS Institute Inc, Cary NC). Data were expressed as mean ± SEM. Data were transformed if it was not normally distributed. All analyses were completed in the mixed procedure using the Kenward-Roger method for degrees of freedom. A p value of < 0.05 was considered significant. Groups were compared using one-way analysis of variance with the model: $y = \mu + group_i + cage_{j(i)} + block_k + \varepsilon_{ijk}$; where μ is the overall mean, $group_i$ is the fixed effect (C26, C26-CPT-11, C26-Cys+CPT-11), $cage_{j(i)}$ is the random effect associated with cage group, $block_k$ is the random effect associated with block (1, 2, 3 or 4), and ε_{ijk} is the random error. If a treatment effect was present, multiple group comparison among the groups was performed using the least significant difference method to compute p-values. Significance between the groups is indicated in all tables with subscript lowercase letters; groups with differing letters are significantly different from each other.

Repeated measures analysis with compound symmetry structure was used for body weight, tumor volume, food intake and water intake with the same model as above, with the addition of period (day) and initial body weight for the body weight analysis.

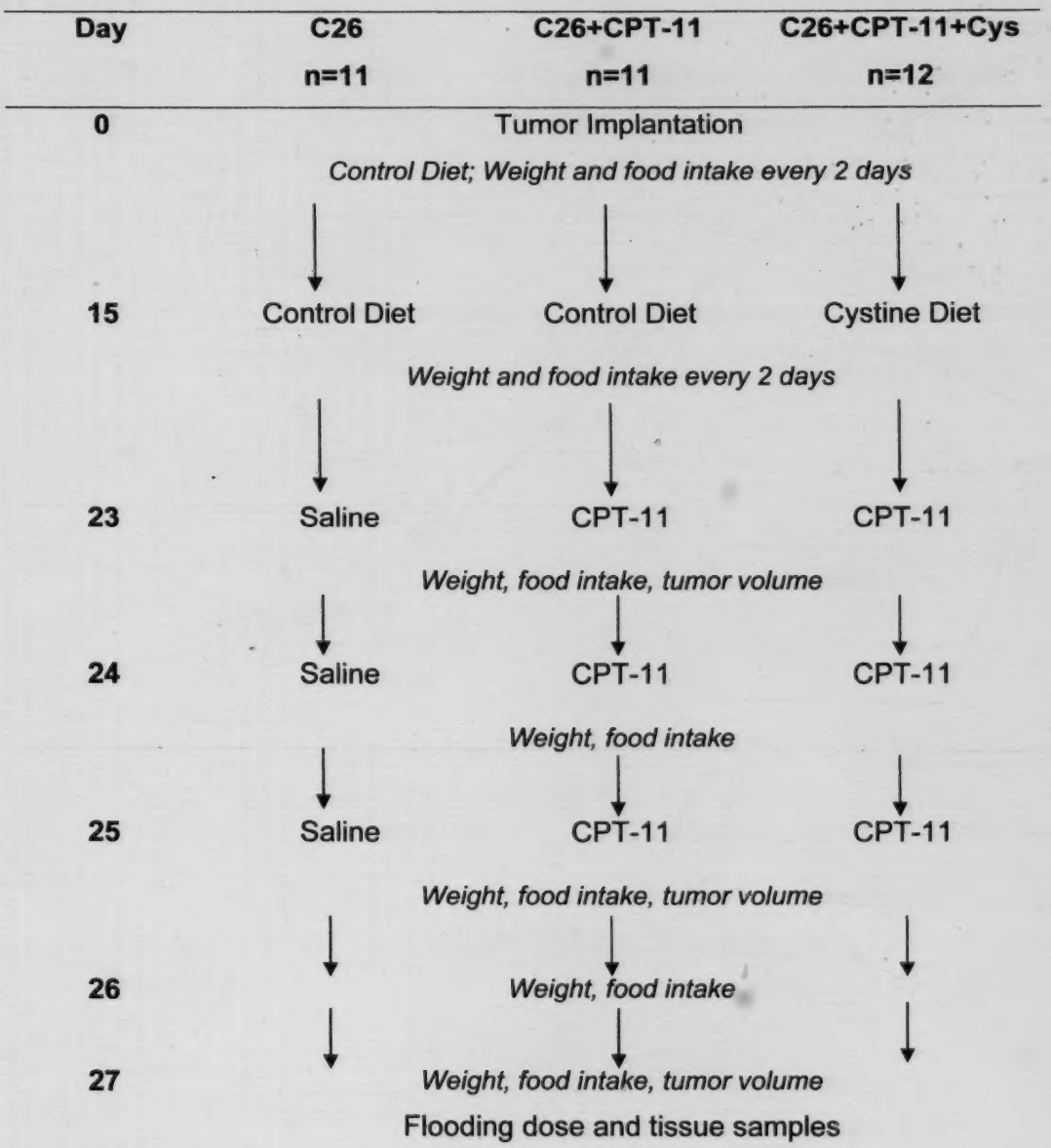

FIGURE 5.1 Study design.

5.3 RESULTS

5.3.1 BODY WEIGHT AND WEIGHT GAIN

Body weight prior to the CPT-11 or saline treatment did not differ among the groups. Body weight (**FIGURE 5.2**) was maintained in the saline treated group. In contrast, body weight decreased in the CPT-11 treated mice ($p<0.0001$). The loss in body weight started 24 h after the first CPT-11 dose and continued throughout the treatment period. Cystine supplementation did not alter the CPT-11-induced changes in body weight.

5.3.2 FOOD INTAKE

Average daily food intake during the first 23 days of tumor growth was similar between all groups (**FIGURE 5.3**). CPT-11 treated mice consumed less food than control mice from day 23 to 27, such that average daily intake during those 5 days was 31% lower for both CPT-11 groups compared to saline treated mice ($p<0.0001$) and before CPT-11 treatment within each group ($p<0.0001$). The average daily food intake was not different between the CPT-11 treated groups.

5.3.3 LIVER AND SPLEEN MASS

CPT-11 treatment increased liver mass relative to non-tumor body weight compared to control mice (C26: 4.9 ± 0.1%; C26+CPT-11: 5.4 ± 0.2%; $p=0.04$). Conversely, relative spleen mass was not affected (C26: 1.0 ± 0.1%; C26+CPT-11: 0.8 ± 0.1%). Cystine supplementation did not affect the CPT-11-induced changes in liver (C26+CPT-11+Cys: 5.2 ± 0.1 %) and spleen (C26+CPT-11+Cys: 0.7 ± 0.1%) mass relative to non-tumor body weight.

5.3.4 MUSCLE MASS AND PROTEIN SYNTHESIS RATE

Gastrocnemius muscle mass (**FIGURE 5.4A**) was reduced by CPT-11 treatment (C26 vs. C26-CPT-11: $p= 0.001$), but relative to non-tumor body weight (**FIGURE 5.4B**), CPT-11 had no effect, indicating that muscle mass loss was proportional to body weight loss. Cystine supplementation did not affect gastrocnemius mass; but it increased mass relative to non-tumor body weight when compared

to mice fed the control diet that received CPT-11 (C26+CPT-11+Cys vs. C26+CPT-11: p=0.04) and saline (C26+CPT-11+Cys vs. C26: p=0.0007). Gastrocnemius fractional protein synthesis rate after CPT-11 treatment (**FIGURE 5.4C**) was not affected by cystine supplementation. Although gastrocnemius fractional protein synthesis rate in either CPT-11 treated group were not different from saline treated mice when compared separately, together the CPT-11 treated groups (C26-CPT-11 + C26-Cys+CPT-11: 2.06 ± 0.24 %/d) had a lower gastrocnemius fractional protein synthesis rate (p=0.049).

5.3.5 TUMOR RESPONSE TO CPT-11

Tumor volume at the start of CPT-11 treatment on day 23 was the same for all groups (C26: 290 46 mm^3; C26+CPT-11: 311 49 mm^3; C26+CPT-11+Cys: 309 63 mm^3). Tumor volume in the saline treated control mice increased from day 23 to 27 (**FIGURE 5.5**; p<0.0001). CPT-11 reduced the change in tumor volume from day 23 to 27 by 57% (C26 vs. C26+CPT-11: p=0.0010). Cystine supplementation during CPT-11 decreased the change in tumor volume by an additional 45% (C26+CPT-11+Cys vs. C26: p<0.001; C26+CPT-11+Cys vs. C26+CPT-11: p=0.01).

5.3.6 TUMOR CYST(E)INE AND GLUTATHIONE

The concentration of total cyst(e)ine in tumor tissue was not affected by CPT-11 treatment or cystine supplementation during treatment (**TABLE 5.1**). Also, the concentration of total, reduced and oxidized glutathione concentration in tumor tissue was not affected by CPT-11 treatment or cystine supplementation.

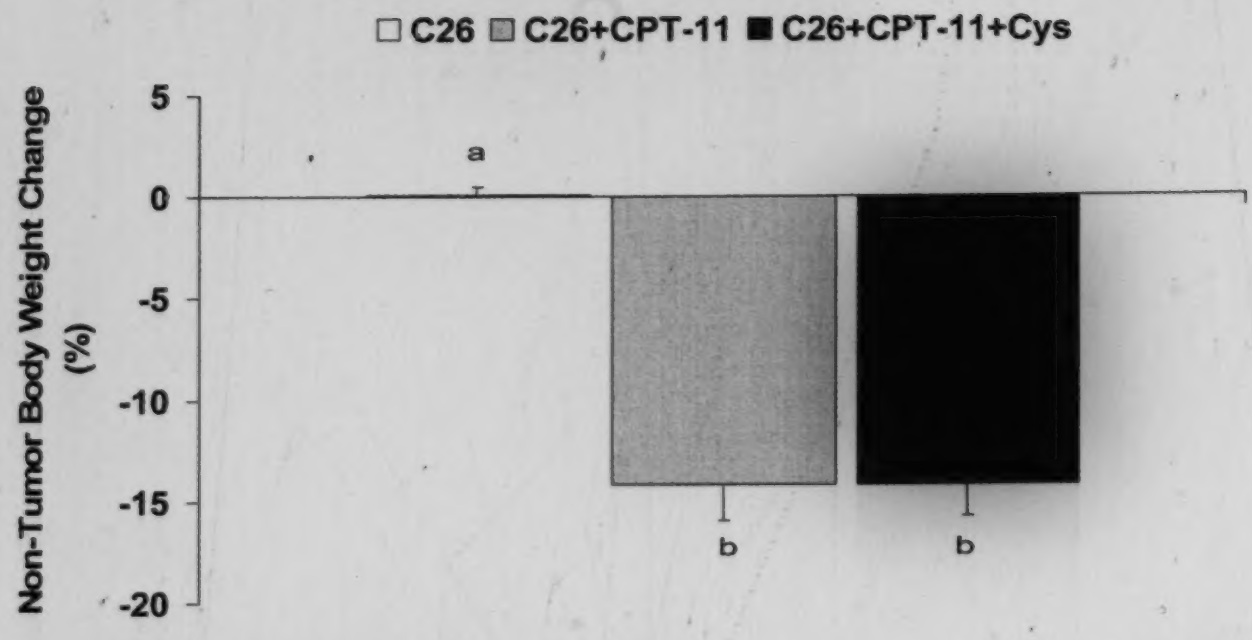

FIGURE 5.2 Percent change in tumor-free body weight from immediately before to 48 hours after three consecutive days of CPT-11 or saline treatment in colon 26 (C26)-bearing mice fed either a control or cystine (Cys) supplemented diet. n=11-12 per group. Groups were compared using ANOVA; significance in multiple group comparisons indicated by different lowercase letters.

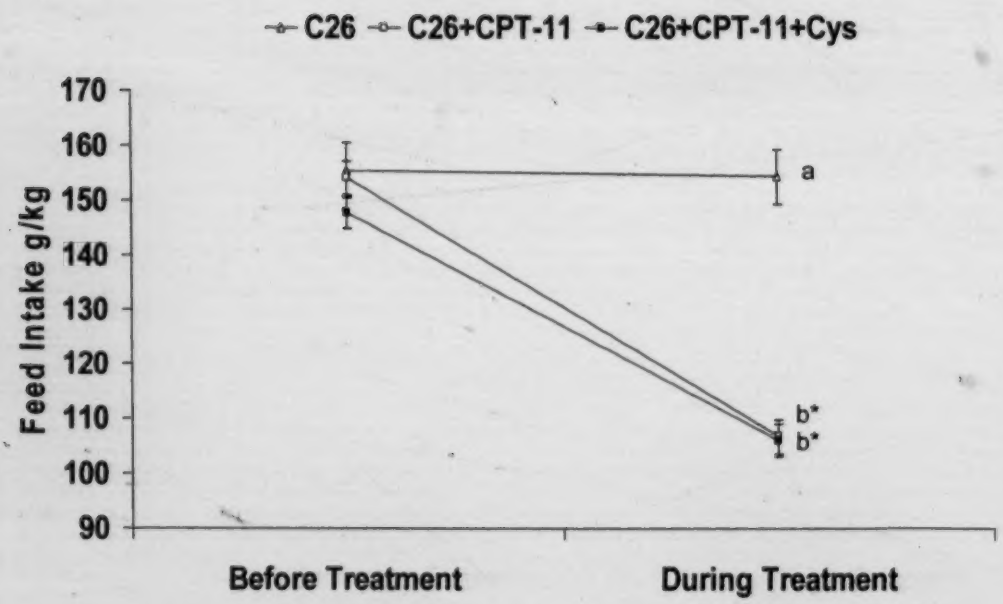

FIGURE 5.3 Food intake before and during treatment with CPT-11 or saline in colon 26 (C26)-bearing mice fed either a control or cystine (Cys) supplemented diet. n=4 cages per group. Groups were compared using ANOVA repeated measures; multiple group differences indicated by different lowercase letters, *indicates significance from before treatment within the group.

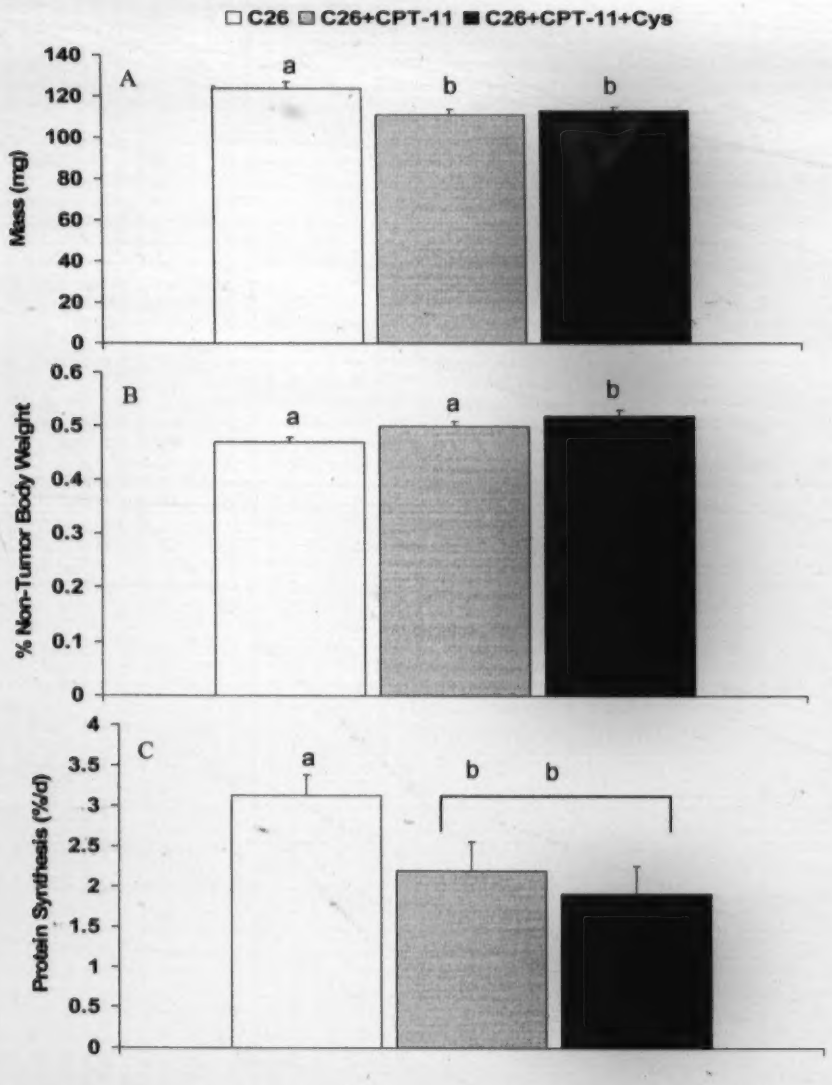

FIGURE 5.4 Gastrocnemius muscle mass (A), percent non-tumor body weight (B) and fractional protein synthesis rate (C) 48 hours following the final CPT-11 or saline treatment in colon 26 (C26)-bearing mice fed either a control or cystine (Cys) supplemented diet. n=11-12 per group for mass and percent non-tumor body weight, n=6 per group for protein synthesis. Groups were compared using ANOVA; significance in multiple group comparisons indicated by different lowercase letters.

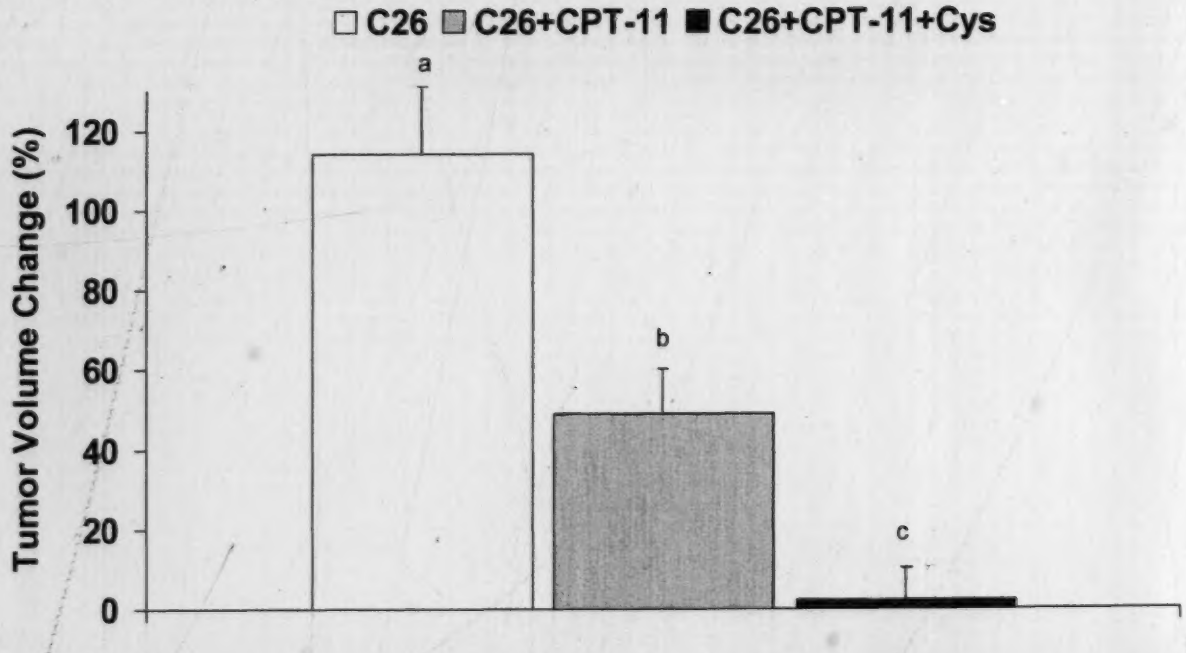

FIGURE 5.5 Percent change in tumor volume from immediately before to 48 hours after three consecutive days of CPT-11 or saline treatment in colon 26 (C26)-bearing mice fed either a control or cystine (Cys) supplemented diet. n=11-12 per group. Groups were compared using ANOVA; significance in multiple group comparisons indicated by different lowercase letters.

TABLE 5.1 Tumor cyst(e)ine and glutathione concentrations 48 hours following CPT-11 or saline treatment in colon 26 (C26)-bearing mice fed either a control or cystine (Cys) supplemented diet.

Mean ± SEM	C26	C26+CPT-11	C26+CPT-11+Cys
	Cyst(e)ine nmol/g		
Total Cysteine	269 ± 8	252 ± 31	265 ± 33
	Glutathione nmol/g		
Total	2553 ± 350	2814 ± 402	2909 ± 262
Reduced	2430 ± 361	2722 ± 514	2881 ± 306
Oxidized	62 ± 27	52 ± 7	35 ± 5
R:O	75 ± 25	54 ± 11	83 ± 12

R:O: reduced to oxidized ratio. n=4-5 per group. Groups were compared using ANOVA; there were no significant differences.

5.4 Discussion

Nutritional supplements in patients with cancer will likely be used in conjunction with anti-tumor therapies. Therefore, we tested the hypothesis that dietary cystine could improve skeletal muscle mass in mice-bearing the C26 tumor treated with the chemotherapeutic agent, CPT-11. Contrary to our hypothesis, dietary cystine supplementation did not prevent the loss of body weight and skeletal muscle mass induced by CPT-11 treatment in tumor-bearing mice. However, cystine supplementation increased the tumor response to CPT-11 treatment.

CPT-11 treatment induced a 14% reduction in weight loss with a proportional reduction in skeletal muscle mass. The effect of CPT-11 treatment on skeletal muscle protein synthesis has not been reported previously. CPT-11 reduced skeletal muscle protein synthesis by 32% compared to saline treated controls. The C26 tumor alone results in a reduction in skeletal muscle protein that starts at a tumor burden less than 2% (Samuels et al., 2001). The fact that CPT-11 reduced skeletal muscle protein synthesis further suggests that chemotherapy treatment contributes to the loss of skeletal muscle mass in cancer, and highlights the importance of nutritional therapies to support skeletal muscle protein anabolism during chemotherapy treatment.

Dietary cystine supplementation did not alter the CPT-11-induced loss of body weight or absolute skeletal mass. The small increase in gastrocnemius mass relative to body weight would unlikely be physiologically beneficial. The reduction in food intake may have limited the effects of cystine, since dietary amino acids would be oxidized during negative energy balance. Cysteine catabolism during first-pass metabolism may have lowered the amount of cysteine that reached systemic circulation and thus skeletal muscle.

The reduction in food intake with CPT-11 treatment would have contributed to the reduction in body weight, skeletal muscle mass and skeletal muscle protein

synthesis. CPT-11 treated mice had a 30% reduction in food intake during the 5 day treatment period. Mice with a 50% reduction in food intake for 7 days had a 30% reduction in skeletal muscle protein synthesis and a 17% reduction in body weight (Svanberg et al., 2000). The length and severity of malnutrition was greater in this study compared to our study, but it indicates that the reduction in food intake had an influence on skeletal muscle protein synthesis.

The most prominent effect of cystine supplementation was a reduction in tumor volume with CPT-11 treatment that was greater than in mice fed the control that received CPT-11. Furthermore, 50% of the cystine supplemented mice had a reduction in tumor volume with CPT-11 treatment compared to only 9% of mice fed the control diet. Although the study was not designed to determine the mechanism of cysteine influence on CPT-11 efficacy, the literature suggests some potential mechanisms. The active metabolite to CPT-11, SN38, is metabolized to the nontoxic SN38-glucuronide by UDP-glucuranyl transferase prior to excretion in bile. Increased intake of dietary sulfur amino acids decreased activity of UDP-glucuranyl transferase in rats (Magdalou et al., 1979). Therefore, the higher dietary cyst(e)ine intake may have delayed the clearance of SN38 and prolonged tumor exposure to SN38. Cyst(e)ine may also improve CPT-11 efficacy through increased apoptosis associated with a reduction in ROS. Although the production of ROS is associated with the toxicity of CPT-11, ROS can also prevent apoptosis through activation of nuclear factor-kappaB (NF-κB) (Kokura et al., 2005). Edaravone, a free radical scavenger, increased SN38-induced apoptosis in C26 tumor cells through a reduction in ROS and NF-κB activation (Kokura et al., 2005). Therefore, the apoptotic actions of CPT-11 do not appear to be dependent on the production of ROS. The increased efficacy of CPT-11 by edaravone was also demonstrated in C26-bearing mice (Kokura et al., 2005).

The impact of cyst(e)ine on the tumor response to combination therapy common in the treatment of colon cancer, specifically CPT-11 and 5-fluorouracil

(5-FU), is not known. Treatment of HCT-15-bearing mice with N-acetyl-cysteine and 5-FU increased tumor apoptotic index and necrotic area with a reduction in tumor volume compared to mice treated with 5-FU alone (Bach et al., 2001), suggesting that cysteine would be beneficial during combination therapy with CPT-11 and 5-FU. Although cysteine supplementation did not alter weight loss or the reduction in skeletal muscle protein synthesis during chemotherapy treatment, the greater reduction in tumor volume warrants further investigation of the usefulness of cysteine as an adjunct to chemotherapy treatment.

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CHAPTER 6. DISCUSSION

6.1 SUMMARY OF STUDIES (FIGURE 6.1)

Chapter 3. A pro-inflammatory tumor that activates protein degradation sensitizes rats to catabolic effects of endotoxin

Hypothesis: Administration of low dose endotoxin will induce an exaggerated catabolic response in skeletal muscle in rats bearing an inflammatory tumor.

Summary of Results: The Yoshida hepatoma reduced muscle mass and increased muscle protein degradation. Although the selected low dose of endotoxin had no impact on muscle protein metabolism in control rats, endotoxin in rats bearing Yoshida hepatoma enhanced weight loss, whole body nitrogen loss and skeletal muscle protein degradation.

Chapter 4. Ibuprofen, but not supplemental cystine, improves skeletal muscle mass in a tumor model of chronic inflammation

Hypothesis: Chronic inflammation in the tumor bearing state contributes to the loss of body weight loss and muscle mass through an increased demand for cysteine. Hence, dietary supplementation with cystine or treatment with an anti-inflammatory agent will reduce muscle wasting associated with tumor growth.

Summary of Results: Ibuprofen treatment in mice bearing the colon 26 tumor partially restored body weight, skeletal muscle mass, skeletal muscle protein synthesis, intramuscular glutathione and systemic cysteine availability. In contrast, dietary supplementation with cystine was not effective. However, cyst(e)ine concentration in plasma and muscle was not increased with supplementation. The effect of cystine supplementation was limited to an increase in tumor cyst(e)ine concentration, but tumor growth and protein synthesis were not affected.

Chapter 5. Dietary cystine supplementation improves the tumor response to irinotecan treatment in mice bearing the colon 26 tumor

Hypothesis: Cystine supplementation will promote the maintenance of body weight and skeletal muscle mass in the tumor-bearing state during chemotherapy treatment without a negative effect on the tumor response.

Summary of Results: Dietary cystine supplementation did not reduce the loss of body weight and skeletal muscle mass induced by treatment with the chemotherapeutic agent, irinotecan, in mice bearing the colon 26 tumor. However, cystine supplementation increased the reduction in tumor volume in response to irinotecan.

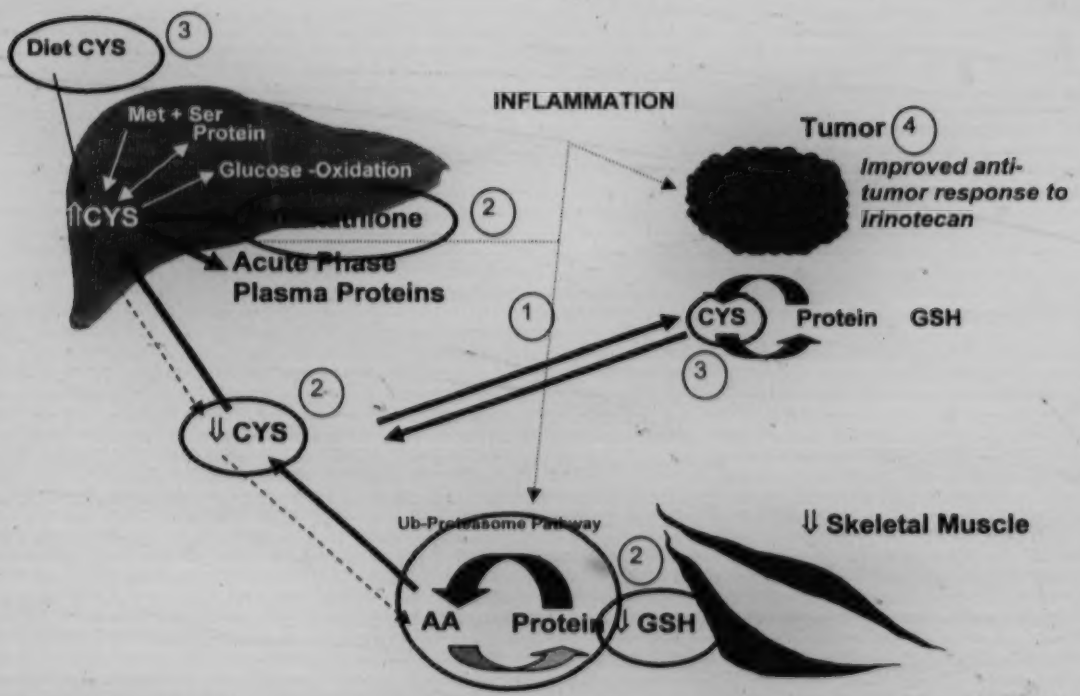

FIGURE 6.1 Summary of studies. **(1)** Increased inflammation elevated skeletal muscle protein degradation (Chapter 3), while inflammation suppression partially restored skeletal muscle protein synthesis (Chapter 4) in the tumor-bearing state. Together these results indicate that inflammation promotes the loss of skeletal muscle protein through increased protein catabolism and reduced protein synthesis in the tumor-bearing state. **(2)** Inflammation in the tumor-bearing state also contributed to lower glutathione concentration in skeletal muscle and liver, and decreased cyst(e)ine concentration in plasma and skeletal muscle (Chapter 4). **(3)** Dietary cystine supplementation did not improve skeletal muscle mass or protein synthesis in the tumor-bearing state; however, cyst(e)ine concentration in plasma and skeletal muscle was also not increased by cystine supplementation (Chapter 4). Cystine supplementation did increase tumor cyst(e)ine concentration, but not tumor growth or protein synthesis (Chapter 4). **(4)** Cystine supplementation increased the tumor response to treatment with the chemotherapeutic agent, irinotecan (Chapter 5).

6.2 Discussion and conclusion

The objectives of this thesis were to critically review the literature on the utilization of amino acids in cancer, establish the specific role of inflammation on skeletal muscle wasting, and determine if an inflammation driven demand for a specific amino acid, cyst(e)ine, limits skeletal muscle protein synthesis in the tumor-bearing state. The following is a discussion of the major findings in the thesis, the clinical relevance, limitations and future directions.

The understanding of amino acid nutrition in the tumor-bearing state is complicated by the use of a variety of model systems. Animal models used to examine mechanisms and therapies in cancer are not likely to translate to the clinical situation unless the type of tumor and dietary design, as well as features of the host response to the tumor are relevant (Xue et al., 2007). The studies in the thesis that examined the efficacy of anti-inflammatory and cystine supplementation (Chapter 4 and 5) used a model system of colon cancer in mice. Colon cancer was chosen since it is a prevalent type of cancer and is associated with one of the highest occurrences of cachexia (American Cancer Society, 2007; Canadian Cancer Society/National Cancer Institute of Canada; Dewys et al., 1980). As well, a systemic IL-6 response is predominant in colon cancer (Belluco et al., 2000; Chung & Chang, 2003) and in the colon tumor model used to examine ibuprofen and cystine in this thesis.

Two different tumor models were used in this thesis. Although anti-inflammatory and cystine treatment was studied in the colon tumor model, the Yoshida heptoma was used to determine the effect of inflammation augmentation on skeletal muscle wasting (Chapter 3). The major finding in the Yoshida hepatoma model was potentiation of skeletal muscle wasting after endotoxin administration. Pilot studies in the colon tumor model (Appendix A) demonstrated that spleen cells from tumor-bearing mice had an elevated IL-6 response to *in vitro* stimulation with endotoxin, suggestive that endotoxin administration *in vivo* would have a similar response in the colon tumor model.

The alteration to skeletal muscle protein metabolism is similar between the colon and Yoshida models. Skeletal muscle protein is reduced by ~40% in both the colon tumor (Chapter 4; Samuels et al., 2001) and Yoshida hepatoma model (Baracos et al., 1995; Strelkov et al., 1989). The degree of proteolysis activation between the models is more difficult to compare because skeletal muscle degradation rate in the colon tumor model has only been measured in one study, and it was an indirect measurement. The Yoshida hepatoma increased proteolysis by 50-100% (Baracos et al., 1995; Strelkov et al., 1989) while proteolysis in the colon tumor model was increased by 125% (Samuels et al., 2001). Ibuprofen treatment increased skeletal muscle protein synthesis in the colon tumor model by 25% (Chapter 4). In comparison, administration of a COX inhibitor in the Yoshida hepatoma completely restored skeletal muscle protein synthesis and degradation to nontumor control levels; however tumor burden was also reduced by 50% (Baracos et al., 1995; Strelkov et al., 1989).

Most studies in amino acid nutrition in the tumor-bearing state are limited to the examination of host x tumor interactions. However, the application to the clinical situation is limited without an examination of the host x tumor x anti-neoplastic therapy. Most cancer patients who will consume a nutritional supplement will receive chemotherapy or radiation at some point. Therefore, it is important that potential interactions are investigated. Cystine supplementation was examined during tumor growth alone (Chapter 4) and during treatment with a chemotherapeutic agent (Chapter 5). The chemotherapeutic agent used, CPT-11, is used as first-line treatment in colorectal cancer; however, it is commonly used in combination therapy with other chemotherapy drugs. Therefore, further research is required to determine the impact of dietary cystine on the tumor response to combination therapy. The greater tumor response to CPT-11 treatment with cystine supplementation was an unexpected finding, but underscores the importance of examining potential interactions.

An issue in the pre-clinical assessment therapies for the treatments of cancer-associated cachexia that was not addressed in this thesis was the effect of age. The rodents used in the studies were young and in a period of rapid growth. However, the average patient with cancer is not in a period of growth and age may impair the responsiveness of skeletal muscle protein synthesis to anabolic stimulation. Older rats required twice as much leucine than young rats to stimulate skeletal muscle protein synthesis to the same level (Dardevet, et al., 2000). Conversely, aged mice had greater sensitivity to an inflammatory stimulus (Tateda et al., 1996); therefore, age may increase the catabolic response to inflammation associated with the C26 tumor or low dose endotoxin. Studies in tumor models using aged rodents are required to examine the effects of age on the metabolic response to inflammation and the effectiveness of anti-cachectic therapies.

The diet used in these studies contained a level and type of fat that is similar to a typical western diet. The importance of diets in preclinical assessment of therapies in cancer has been demonstrated in the use of anti-inflammatory drugs and cancer prevention where the use of standard rodent diets rather than high fat diets has been implicated in the lower efficacy of COX inhibitors in the prevention of cancer in clinical application compared to preclinical models (Rao & Reddy, 2004). The ratio of n-6 and n-3 fatty acid in the diet is particularly important in the examination of inflammation because the ratio of the essential fatty acids can modulate inflammation through alterations in cell-membrane composition and function. The ratio of n-6 to n-3 fatty acids in the typical western diet of 15-17 is considered one that would facilitate inflammation (Simopoulos, 2006). An imbalance in eicosanoid production after stimulation is a mechanism of increased inflammation with a high n-6 to n-3 fatty acid intake (Calder, 2003). The long chain n-6 and n-3 fatty acids incorporated into cell membranes are the precursors for the synthesis of eicosanoids. However, the inflammatory potency of the eicosanoids produced from the n-6 fatty acid arachidonic is greater then the eicosanoids produced from the n-3 fatty acid

eicosapentaenoic (Calder, 2003). Since the relative amount of n-6 and n-3 fatty acids incorporated into cell membranes is determined by dietary intake, a high n-6 to n-3 fatty acid ratio of 18 was used to facilitate an inflammatory state.

Nutritional modulation of inflammation has been studied as an alternative to the use of anti-inflammatory drugs. Nutrients with anti-inflammatory properties include cysteine through the synthesis of glutathione and the n-3 fatty acids through alterations in eicosanoid production. However, the comparison of cystine and ibuprofen indicates that the relative efficacy of the COX inhibitor was superior to nutritional modulation with cystine (Chapter 4). Additionally, cystine supplementation eliminated the positive effects of ibuprofen on body weight and skeletal muscle mass. Few other studies have directly compared the efficacy of anti-inflammatory drugs with nutritional modulation of inflammation. A study in mice with lung inflammation induced by aerosolized endotoxin found that dexamethasone treatment was a more effective anti-inflammatory and anti-oxidant than N-acetyl-cysteine (Rocksen et al., 2000). Supplementation with n-3 fatty acids has been used in cancer patients (Colomer et al., 2007); however a direct comparison to anti-inflammatory drugs has not been examined in the treatment of cancer-associated cachexia. Although a high fish oil diet suppressed the development of azoxymethane-induced colon cancer in rats, the efficacy was lower than celcoxib treatment (Reddy et al., 2005), suggesting that the anti-inflammatory response to n-3 fatty acids was lower than the COX inhibitor in this study. The complicated metabolism and broad actions of nutrients compared to the specific actions of anti-inflammatory drugs is likely the reason for the differences in efficacy. As encountered in the cystine supplementation studies, the ultimate fate of a nutrient depends on a number of factors including utilization in first pass metabolism, which may limit the nutrient from reaching the target tissue.

Dietary cystine supplementation was used in this thesis to determine if cysteine was limiting skeletal muscle protein synthesis in the tumor-bearing state

(Chapter 4 and 5). However, some aspects of the diet design may have influenced the ineffectiveness of cystine supplementation. The methionine levels in our diets were designed to supply the same amount methionine as the American Institute of Nutrition (AIN)-76A semi-purified diet for growing rodents (American Institute of Nutrition, 1977; National Research Council 1995). However, the minimum methionine requirement has not been empirically determined in mice. The determination of requirements in mice is complicated by the numerous strains with different rates of growth (National Research Council, 1995). The level of methionine is important because an excess in dietary methionine would increase methionine flux through the transulphuration pathway. To avoid this limitation, the minimum dietary methionine requirement specific for Balb/c mice fed our background diet would need to be determined. Another factor that needs to be studied is the optimal dietary cysteine source that would increase plasma and tissue cyst(e)ine levels. Cysteine and cystine are toxic at high levels toxic (Dilger, et al., 2007); therefore, alternatives such as the cysteine precursor N-acetyl-cysteine or a high-cysteine protein should be considered.

The use of amino acid supplementation to identify limiting amino acids is common in tumor-bearing models, animal models of other disease states and in the clinical setting; however, this approach has several limitations. Dietary provision of amino acids does not necessarily increase systemic availability, as was the case with cystine, which can result in the misinterpretation of an amino acid as not limiting. A different approach to identify amino acids that limit protein synthesis is the plasma amino acid response to systemic administration (Cynober, 2003). The plasma amino acid response method has been used to manipulate parenteral amino acid formulation to meet the specific needs of hospitalized patients in an intensive care unit (Berard et al., 2002). The advantages to the plasma amino acid response method are that all limiting amino acids can be identified at once and the relative degree of limitation can be determined.

The role of inflammation was studied in the tumor-bearing state at three different levels of inflammation severity. Inflammation associated with tumor growth was enhanced with endotoxin (Chapter 3) and reduced with anti-inflammatory drug treatment (Chapter 4). The modifications in skeletal muscle protein balance were related to the level of inflammation, indicating that inflammation contributes to skeletal muscle wasting. The elevation of skeletal muscle proteolysis in response to endotoxin suggests that periods of acute inflammation, such as during infections, enhance the loss of skeletal muscle mass in patients with cancer. Infections are common and often the cause of death in patients with cancer (Inagaki et al., 1974; Nagy-Agren & Haley, 2002). The role of an exaggerated metabolic response to acute inflammatory stimuli in the tumor-bearing state in the mortality associated with infections in cancer is an area that requires further investigation.

The effectiveness of ibuprofen in the maintenance of body weight and skeletal muscle mass during tumor growth supports the use of anti-inflammatory drugs in the treatment of cachexia in cancer. Although skeletal muscle mass and protein synthesis were increased by ibuprofen treatment, protein synthesis rate was still below the non-tumor-bearing reference level (Chapter 4). Additional therapies, including adequate amino acids and anabolic stimulation, are likely required in addition to inflammation suppression to fully support maintenance of lean body mass. The effect of COX inhibitors during chemotherapy treatment were not studied in this thesis; however a COX-2 specific inhibitor improved the efficacy of CPT-11 treatment in mice bearing the colon 26 tumor (Trifan et al., 2002). Therefore, the use of anti-inflammatory drugs may have benefits in cancer beyond the treatment of cachexia.

The studies of this thesis have established the specific role of inflammation on skeletal muscle loss in the tumor-bearing state. The heightened metabolic response to acute inflammatory stimulation in the tumor-bearing state suggests

that infections contribute substantially to the loss of body weight and skeletal muscle wasting in patients with cancer. However, the effectiveness of the COX inhibitor, ibuprofen, indicates that management of inflammation is effective in partially restoring skeletal muscle protein synthesis. Although dietary cystine supplementation was not effective in the treatment of skeletal muscle loss, further studies is required to determine if cysteine demand is increased in the tumor-bearing state.

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APPENDIX A. PILOT STUDY TO CHARACTERIZE MUSCLE WASTING AND INFLAMMATORY MEDIATORS IN THE COLON 26 MURINE TUMOR MODEL

A.1 PURPOSE OF PILOT STUDY

The animal models used for the pre-clinical assessment of therapies in the tumor-bearing state need to be clinically relevant. The objective of this pilot study was to characterize the development of cachexia and pro-inflammatory cytokine response in mice bearing the colon 26 (C26) adenocarcinoma fed a semi-purified diet used by our research group. The level and type of fat in rodent diets can influence tumor growth (Birt, 1997); therefore, it is important to use diets similar to what is consumed in patients with cancer. The tumor and host pro-inflammatory cytokine release was also examined to further characterize the inflammatory response in the C26 tumor.

A.2 MATERIAL AND METHODS

ANIMALS AND DIET Male Balb/c mice were obtained from Charles River (St. Constant, Quebec). Mice were kept in plastic shoebox cages with wire tops (3-5 mice per cage) in a temperature and humidity controlled room on a 12 hour light-dark cycle. All studies were conducted in accordance to Canadian Council on Animal Care Guidelines and were approved by the institutional Animal Policy and Welfare Committee. Mice had free access to water and feed. Mice were fed a modified AIN-76 semi-purified diet (**TABLE A.1**) that met the nutrient requirements for mice (National Research Council, 1995). The fat content of the diet supplied 40% of energy and had a low polyunsaturated:saturated ratio (0.4) and a high n-6:n-3 ratio (18).

TUMOR Frozen vials of C26 tumor cells obtained from National Cancer Institute Tumor Repository (Frederick, MD) were cultured in RPMI 1640 with glutamine, antibiotic-antimycotic (100units penicillin, 0.25ug streptomycin, 0.25ug amphotericin B per 100mL), and fetal bovine serum (10% v/v). Gibco (Invitrogen Canada Inc., Burlington, ON) cell culture products were used unless

stated otherwise. Vials containing $1x10^6$ cultured cells were slowly frozen at -20°C overnight followed by liquid nitrogen storage. Before utilization in the mice used in the pilot study, the C26 tumor cells from stock underwent two passages in mice. Tumor cells from the second passage were injected (1.5×10^6 cells s.c.) into n=8 mice (21 days old). Three Balb/c mice were used as non-tumor-bearing controls.

SAMPLING AND CELL PREPARATION Mice were weighed every other day and fresh feed was given daily. Fourteen days after tumor implantation, all mice were anesthetized with sommatol (i.p) and cardiac puncture was performed to obtain a blood sample followed by cervical dislocation. Liver and muscles (tibialis anterior and gastrocnemius) were dissected and weighed. Spleen and tumors were removed and weighed aseptically before cells were isolated under sterile conditions. Cells (1×10^6 tumor cells, 3×10^6 spleen cells) were incubated in media. Two samples of spleen cells were prepared for each mouse: one sample contained only media and the second sample had LPS (25mg E coli O55:B5/L, Sigma, St. Louis, Missouri) added to the media. One tumor sample from each tumor-bearing mouse was prepared with media only. The samples were incubated for 48 hours at 37°C with 7%CO_2. The supernatants were frozen at -80 °C until analysis.

INTERLEUKIN-6 (IL-6) AND TUMOR NECROSIS FACTORα (TNFα) ANALYSIS ELISA was performed to determine IL-6 and TNFα levels in plasma and the media using mouse specific kits (BD Biosciences, Mississauga, ON). Cytokine release was calculated based on the pg cytokine per million cells incubated. The stimulation index was calculated by the difference between LPS-stimulated and unstimulated cytokine release divided by the unstimulated release.

STATISTICAL ANALYSIS Group data is presented as mean ± SEM. SAS (Version 8.2, SAS Institute, Cary, North Carolina) was used for all statistical analysis. Data were transformed if it was not normally distributed. One-way ANOVA was

performed in the mixed procedure to compare tumor-bearing bearing group to the non-tumor controls. The model used included tumor as the fixed effect and cage as a random effect. Body weight was assessed using repeated measures ANOVA in the mixed procedure. Significance was considered at $p<0.05$.

A.3 Results

Body weight (Figure A.1) Initial body weight was not different between the non-tumor control and C26 groups. The non-tumor bearing mice had continual weight gain over the 14 days; except for day 6 to 8 when weight was maintained. The C26 mice gained weight up to day 6, and body weight was stable thereafter. Compared to the non-tumor bearing controls, the C26-bearing mice had a lower body weight starting 10 days after tumor implantation (day10 $p=0.03$, day12 $p=0.01$, day14 $p=0.006$). The overall effect of C26 was a reduction in body weight during the 14 days of tumor growth ($p=0.04$).

Tissue weights (Table A.2) Tumor weights and burden (percent body weight) had a large variation which will need to be taken into consideration during future use of the model. Fourteen days after implantation of 1.5×10^6 C26 cells the average tumor weight was 823 ± 165 mg with a range of 124-1611 mg. The average tumor burden for the C26 mice was $3.7 \pm 0.7\%$ of body weight (range: 0.5-6.9%). Mice bearing the C26 tumor had significantly lower gastrocnemius mass; however, there was no difference relative to body weight or non-tumor body weight, indicating that the muscle loss of proportional to the reduction in body weight. Liver absolute and relative mass was not affected by the C26 tumor. However, spleen absolute and relative mass was increased in the C26-bearing mice. Since the number of spleen cells isolated per milligram of tissue was not affected by C26 tumor (Non-tumor controls: $1.27 \times 10^5 \pm 0.17 \times 10^5$ cells/mg spleen tissue C26: $1.35 \times 10^5 \pm 0.10 \times 10^5$ cells/mg spleen tissue, $p=0.7$), the increase in spleen mass was from tissue growth rather than a change in fluid balance.

PRO-INFLAMMATORY CYTOKINES (TABLE A.3 AND A.4) The C26 tumor increased plasma IL-6 concentrations 10-fold. Plasma TNFα was lower in the C26-bearing mice than controls; however, both C26 and control levels were below or near the detectable limit of the assay (5pg/mL). Cells isolated from C26 tumor tissue and incubated for 48 hours released both IL-6 and TNFα. While IL-6 release from spleen cells was 75-fold higher in C26-bearing mice than non-tumor controls, TNFα release was only 2-fold higher.

LPS, a B cell and macrophage mitogen, was used to stimulate spleen cells. LPS stimulation increased IL-6 spleen cell release in non-tumor control ($p=0.03$) and C26-bearing mice ($p=0.0001$). Per million cells, spleen cells from C26-bearing mice had a 24-fold higher IL-6 release when incubated with LPS for 48 hours compared to control. Despite higher release of IL-6 in spleen cells from C26-bearing mice, the stimulation index was lower compared to non-tumor control indicating a reduction in responsiveness. However, the absolute difference between the unstimulated and stimulated IL-6 release was 100-fold higher in the C26-bearing mice compared to non-tumor controls. The higher absolute increase in IL-6 release is suggestive that the systemic response to acute stimulation could be higher in C26 mice compared to non-tumor controls. In contrast to IL-6 response, the TNF response to LPS stimulation was not affected by the C26 tumor. LPS stimulation increased TNFα spleen cell release from C26-bearing mice ($p=0.0006$), but not non-tumor controls ($p=0.06$). However, TNFα release per million LPS stimulated spleen cells, stimulation index and absolute difference in C26-bearing mice were not different from non-tumor controls.

TABLE A.1 Composition of the diet.

Ingredient	Grams per kg Diet
Casein	240
Cornstarch	150
Cellulose	50
Sucrose	299
Calcium Carbonate	3.6
L-Methionine	3.6
Mineral Mix (AIN-76)	42
Vitamin Mix (AIN-76)	12
Sterine Flakes	121.4
Sunfower Oil	74.6
Flaxseed Oil	4

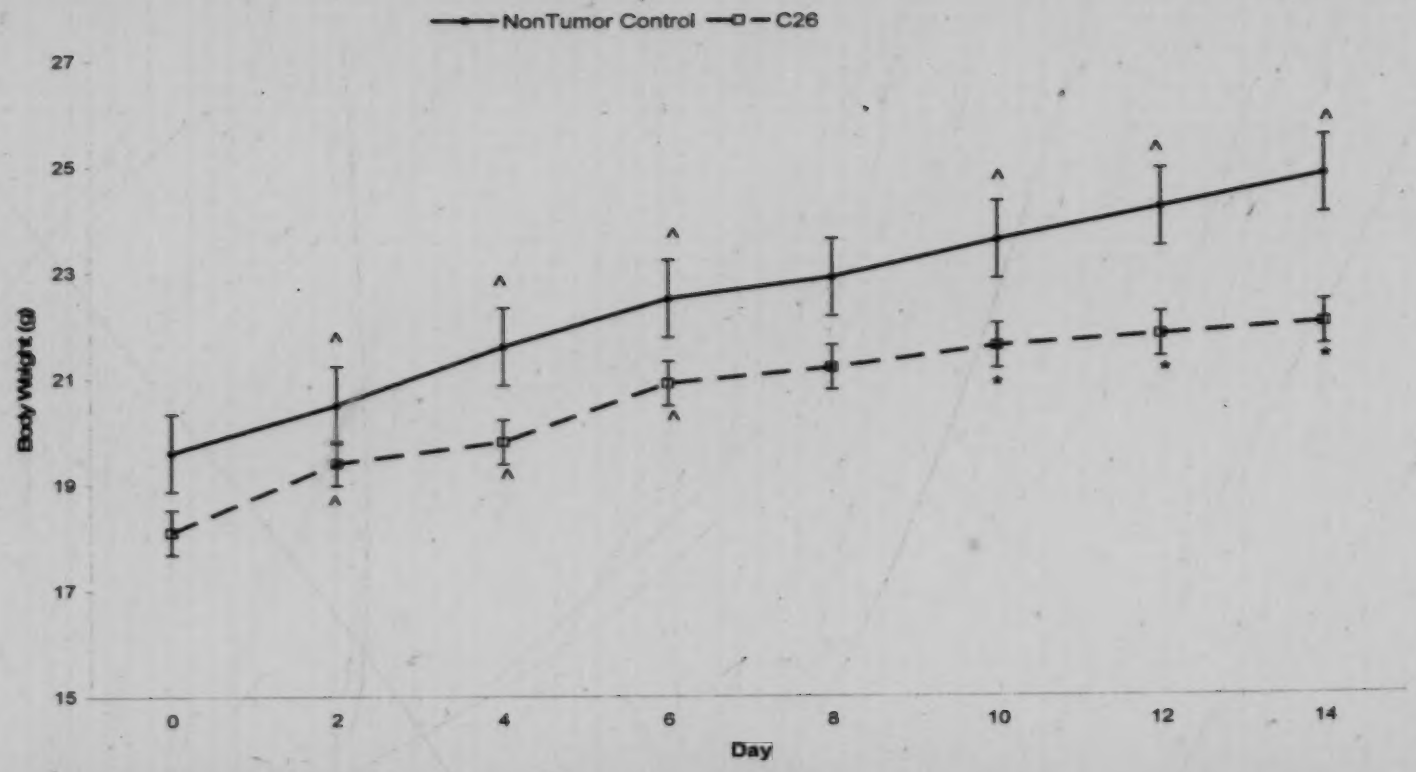

FIGURE A.1 Body weight in non-tumor control and colon 26 (C26)-bearing mice. C26 tumor was implanted on day 0. Points are group means and error bars are SEM. *Significantly different from non-tumor controls ($p<0.05$) repeated measures ANOVA; ^ significantly different from previous time point within that group ($p<0.05$ repeated measures ANOVA).

TABLE A.2 Muscle, liver and spleen weight in non-tumor control and colon 26 (C26)-bearing mice 14 days after tumor implantation.

Mean ± SEM	Control	C26	Control vs. C26 *P* value
	Gastrocnemius Muscle		
mg	129 ± 2	108 ± 2*	0.0015
% Body Weight	0.52 ± 0.01	0.49 ± 0.01	0.1464
%NT Body Weight	0.52 ± 0.01	0.51 ± 0.01	0.7058
	Liver		
mg	1347 ± 49	1159 ± 68	0.1927
% Body Weight	5.43 ± 0.20	5.27 ± 0.28	0.7924
%NT Body Weight	5.43 ± 0.20	5.49 ± 0.32	0.9230
	Spleen		
mg	111 ± 8	185 ± 12*	0.0083
% Body Weight	0.45 ± 0.03	0.84 ± 0.06*	0.0043
%NT Body Weight	0.45 ± 0.03	0.88 ± 0.06*	0.0048

NT: non-tumor. * Significantly different from non-tumor controls.

TABLE A.3 Interleukin 6 (IL-6) and tumor necrosis factor (TNFα) in non-tumor control and colon 26 (C26)-bearing mice 14 days after tumor implantation.

Mean ± SEM	Control	C26	Control vs. C26 *P* value
	Plasma pg/mL		
IL-6	29 ± 19	252 ± 54*	0.0154
TNFα	7 ± 3	0 ± 0	0.0027
	Tumor Tissue Release pg/10^6 cells		
IL-6		14 ± 3	
TNFα		3 ± 1	
	Unstimulated Splenocyte Release pg/10^6 cells		
IL-6	5 ± 1	376 ± 57*	0.0103
TNFα	30 ± 3	61 ± 3*	0.0019
	LPS -Stimulated Splenocyte Release pg/10^6 cells		
IL-6	26 ± 5^	630 ± 76*^	0.0022
TNFα	138 ± 29	291 ± 37^	0.0799

3 x 10^6 spleen cells were incubated for 48hrs in media with (stimulated) and without (unstimulated) LPS. 1 x 10^6 cells from tumor tissue were incubated for 48hrs in media. *Significantly different from non-tumor controls; ^ significantly different from unstimulated ($p<0.05$).

TABLE A.4 Change in Interleukin 6 (IL-6) and tumor necrosis factor (TNFα) release with lipopolysacchride stimulation of spleen cells isolated from non-tumor control and colon 26 (C26)-bearing mice 14 days after tumor implantation.

Mean (SEM)	Control	C26	Control vs. C26 *P* value
	IL-6		
Stimulation Index	4.7 ± 0.5	0.8 ± 0.2*	<0.0001
Difference pg/10^6 cells	22 ± 4	255 ± 33*	0.0219
	TNFα		
Stimulation Index	3.6 ± 0.7	4.0 ± 0.8	0.8495
Difference pg/10^6 cells	108 ± 27	230 ± 39	0.1689

Stimulation Index = (Stimulated / Unstimulated pg per cell) / Unstimulated pg per cell. Difference pg/10^6 cells = Stimulated pg per million cells – Unstimulated per million cells. 3 x 10^6 spleen cells were incubated for 48hrs in media with (stimulated) and without (unstimulated) LPS. *Significantly different from non-tumor controls.

A.4 SUMMARY

The C26 tumor in this pilot study produced cachexia and inflammatory response that is similar to other studies that have used the model (Fujimoto-Ouchi et al., 1995; Matsumoto et al., 1999; Samuels et al., 2001; Tanaka et al., 1989; Tanaka et al., 1990; Yasumoto et al., 1995). We have demonstrated that inflammation associated with the growth of the C26 tumor is derived from tumor and host release of mediators. Both tumor and host derived inflammatory mediators can contribute to systemic inflammation; however, the higher spleen release suggests that the host derived response is to a higher degree. In agreement with our finding of increased spleen cells cytokine release, the C26 tumor increased the mRNA expression for TNFα and IL-6 in spleen (Yasumoto et al., 1995). Although the lower LPS stimulation index for IL-6 release from spleen cells suggests an impaired ability to respond, there is an elevation in absolute IL-6 release in C26-bearing mice. Considering the increased spleen weight, the potential exists for a substantial IL-6 response to acute stimulation in C26-bearing mice. The C26 model will be used to study the contribution of inflammation in muscle wasting and alterations in amino acid requirements.

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APPENDIX B. SUPPLEMENTAL DATA FOR CHAPTER 4

This appendix includes the complete amino acid analysis for plasma, liver, gastrocnemius muscle, and tumor (Table B1 to B4) completed in reference nontumor-bearing mice and C26-bearing mice fed the control or cystine supplemented diet with and without ibuprofen. For details of the amino acid analysis see Chapter 4, Section 4.2.6.2. Table B.5 provides details of food intake including cyst(e)ine, methionine and total sulfur amino acid intake.

TABLE B.1 Free amino acids in plasma from reference mice and mice bearing the colon 26 (C26) tumor fed a control or cystine (Cys) diet with and without ibuprofen (Ibu).

Mean ± SEM nmol/ml	Reference	C26	C26+Cys	C26+Ibu	C26 Cys+Ibu
		Dietary Essential Amino Acids			
Histidine	75 ± 4	70 ± 5	70 ± 5	62 ± 4	75 ± 3
Isoleucine	65 ± 10	55 ± 9[a,b]	71 ± 13[a]	44 ± 4[b]	53 ± 8[a,b]
Leucine	88 ± 13	91 ± 18[a,b]	106 ± 20[a]	66 ± 7[b]	81 ± 15[a,b]
Lysine[B]	266 ± 41	247 ± 12[a,b]	304 ± 45[a]	194 ± 26[b]	239 ± 15[a,b]
Methionine	62 ± 8*	31 ± 2	40 ± 6	32 ± 5	42 ± 5
Phenylalanine	590 ± 37	661 ± 51	675 ± 57	672 ± 60	666 ± 53
Threonine	194 ± 18	160 ± 20	206 ± 34	138 ± 17	171 ± 12
Tryptophan	74 ± 3*	50 ± 7	43 ± 1	45 ± 3	55 ± 6
Valine	187 ± 29	132 ± 34	183 ± 36	103 ± 14	130 ± 22
		Dietary Non-Essential Amino Acids			
Alanine	294 ± 34	236 ± 16	271 ± 46	201 ± 16	237 ± 14
Arginine	88 ± 8	91 ± 16	102 ± 9	74 ± 10	82 ± 5
Asparagine[C]	40 ± 4*	27 ± 2[a,b]	26 ± 3[a,b]	24 ± 1[a]	32 ± 2[b]

TABLE B.1 continued

Mean ± SEM nmol/ml	Reference	C26	C26+Cys	C26+Ibu	C26+Cys+Ibu
Aspartate	13 ± 2	14 ± 1	13 ± 2	16 ± 3	10 ± 1
Citrulline[B]	59 ± 4*	80 ± 7[a]	77 ± 8[a]	54 ± 4[b]	63 ± 4[a,b]
Cyst(e)ine[C]	250 ± 33*	182 ± 14[a]	199 ± 13[a,b]	228 ± 22[b]	194 ± 19[a]
Homocyst(e)ine	14 ± 1	16 ± 1	14 ± 1	14 ± 1	12 ± 1
Glutamate[A]	42 ± 7	59 ± 8[a]	40 ± 7[a,b]	50 ± 8[a,b]	35 ± 5[b]
Glutamine	429 ± 39	463 ± 25	418 ± 38	446 ± 35	466 ± 14
Glycine	185 ± 11	176 ± 11	152 ± 13	184 ± 22	177 ± 19
Ornithine	47 ± 4	61 ± 6	50 ± 8	45 ± 9	43 ± 8
Serine	136 ± 10*	84 ± 6	88 ± 9	81 ± 7	92 ± 6
Taurine	358 ± 66	477 ± 67	547 ± 23	493 ± 63	466 ± 34
Tyrosine[B]	323 ± 24*	131 ± 20[a,b]	103 ± 6[a]	154 ± 27[a,b]	184 ± 15[b]

Note: Flooding dose of phenylalanine given 15 min before sampling. n=5-10. Reference group compared to C26 using ANOVA; significance indicated by asterisk (*). C26-bearing groups compared using factorial ANOVA; significance indicated by uppercase letters for main effects (A: cys, B: ibu, C: interaction) and by different lowercase letters for between groups.

TABLE B.2 Free amino acids in liver from reference mice and mice bearing the colon 26 (C26) tumor fed a control or cystine (Cys) diet with and without ibuprofen (Ibu).

Mean ± SEM nmol/g	Reference	C26	C26+Cys	C26+Ibu	C26+Cys+Ibu
		Dietary Essential Amino Acids			
Histidine					
Isoleucine	156 ± 19	196 ± 28	249 ± 30	175 ± 21	174 ± 22
Leucine	315 ± 41	419 ± 61	510 ± 60	388 ± 57	409 ± 62
Lysine[B]	511 ± 55	891 ± 145[a,b]	1282 ± 145[a]	664 ± 93[b]	643 ± 68[b]
Methionine	83 ± 14	124 ± 19	122 ± 16	114 ± 24	139 ± 25
Phenylalanine[B]	420 ± 45*	961 ± 68[a,b]	1004 ± 127[a]	620 ± 94[b,c]	561 ± 77[c]
Threonine	374 ± 49	347 ± 40	424 ± 50	365 ± 58	478 ± 68
Tryptophan	192 ± 7	200 ± 12	192 ± 23	189 ± 6	179 ± 11
Valine	302 ± 41	389 ± 55	506 ± 82	329 ± 51	345 ± 48
		Dietary Non-Essential Amino Acids			
Alanine	2005 ± 134	1900 ± 152	1746 ± 16	2018 (n=1)	1916 ± 94
Arginine	551 ± 55	468 ± 20	411 ± 61	443 ± 42	482 ± 50
Asparagine	232 ± 25	351 ± 72	422 ± 62	314 ± 58	347 ± 61

TABLE B.2 continued

Mean ± SEM nmol/g	Reference	C26	C26+Cys	C26+Ibu	C26+Cys+Ibu
Aspartate	411 ± 24	629 ± 50[a,b]	902 ± 147[b]	579 ± 51[a]	567 ± 56[a]
Citrulline	42 ± 6	57 ± 4[a]	100 ± 15[b]	57 ± 11[a,b]	58 ± 9[a]
Cyst(e)ine[B]	153 ± 22*	224 ± 16	219 ± 24	207 ± 33	197 ± 18
Homocyst(e)ine	35 ± 10	43 ± 5	40 ± 5	43 ± 11	45 ± 8
Glutamate	1605 ± 183	1749 ± 278	2224 ± 110	1960 ± 250	1797 ± 179
Glutamine	4739 ± 244	4167 ± 298	3943 ± 122	4409 ± 191	4446 ± 287
Glycine	1966 ± 176	1522 ± 134	1388 ± 158	1580 ± 157	1719 ± 207
Ornithine	309 ± 39	492 ± 86	885 ± 161	471 ± 80	456 ± 74
Serine	291 ± 28	288 ± 62	368 ± 78	326 ± 67	396 ± 91
Taurine	9145 ± 469	9634 ± 342	9264 ± 74	9143 ± 258	9251 ± 390
Tyrosine	835 ± 65	751 ± 176	588 ± 29	795 (n=1)	785 ± 94

Note: Flooding dose of phenylalanine given 15 min before sampling. Glutamine and histidine peak not separated, taurine, alanine and tyrosine peaks not separated in all samples. n=6-8. Reference group compared to C26 using ANOVA; significance indicated by asterisk (*). C26-bearing groups compared using factorial ANOVA; significance indicated by uppercase letters for main effects (A: cys, B: ibu, C: interaction) and by different lowercase letters for between groups.

TABLE B.3 Free amino acids in gastrocnemius muscle from reference mice and mice bearing the colon 26 (C26) tumor fed a control or cystine (Cys) diet with and without ibuprofen (Ibu).

Mean ± SEM nmol/g	Reference	C26	C26+Cys	C26+Ibu	C26+Cys+Ibu
		Dietary Essential Amino Acids			
Histidine[C]	149 ± 5	134 ± 7[a]	155 ± 5[b]	157 ± 5[b]	149 ± 6[a,b]
Isoleucine[B]	79 ± 5	73 ± 6[a,b]	98 ± 15[a]	68 ± 6[b]	62 ± 5[b]
Leucine[B]	133 ± 10	128 ± 13[a,b]	177 ± 30[a]	112 ± 10[b]	109 ± 9[b]
Lysine	312 ± 14	589 ± 75	654 ± 34	576 ± 56	661 ± 91
Methionine	105 ± 10	75 ± 7	88 ± 9	81 ± 10	73 ± 7
Phenylalanine	592 ± 26	650 ± 31	622 ± 65	546 ± 38	603 ± 30
Threonine	379 ± 21	295 ± 28	386 ± 33	341 ± 30	307 ± 23
Tryptophan	95 ± 3	104 ± 8	101 ± 6	99 ± 3	95 ± 4
Valine	237 ± 20	214 ± 29[a,b]	263 ± 45[a]	183 ± 19[a,b]	174 ± 15[b]
		Dietary Non-Essential Amino Acids			
Alanine	1700 ± 72	1598 ± 98	1576 ± 70	1682 ± 69	1545 ± 61
Arginine	1006 ± 38	805 ± 47	871 ± 41	977 ± 28	899 ± 52
Asparagine	146 ± 7	115 ± 7	139 ± 10	128 ± 7	126 ± 9

TABLE B.3 continued

Mean ± SEM nmol/g	Reference	C26	C26+Cys	C26+Ibu	C26+Cys+Ibu
Aspartate	233 ± 15*	117 ± 11	134 ± 12	138 ± 11	119 ± 7
Citrulline^{B}	119 ± 8*	162 ± 6^{a}	172 ± 8^{a}	140 ± 6^{b}	135 ± 9^{b}
Cyst(e)ine	23 ± 3	34 ± 2	39 ± 6	31 ± 2	34 ± 6
Glutamate^{B}	546 ± 29*	369 ± 19^{a}	371 ± 18^{a}	455 ± 23^{b}	402 ± 24^{a,b}
Glutamine	1926 ± 78	1675 ± 84	1884 ± 181	1744 ± 61	1703 ± 65
Glycine^{B}	2159 ± 164	1404 ± 69^{a}	1411 ± 68^{a}	1774 ± 74^{b}	1556 ± 85^{a,b}
Ornithine	70 ± 5	89 ± 12	81 ± 2	79 ± 6	81 ± 6
Serine	484 ± 24*	271 ± 12	314 ± 21	316 ± 14	292 ± 13
Taurine	3455 ± 75	3833 ± 252	3727 ± 208	3625 ± 114	3646 ± 202
Tyrosine^{B}	340 ± 18*	187 ± 14^{a}	210 ± 14^{a,b}	239 ± 11^{b}	224 ± 10^{a,b}

Note: Flooding dose of phenylalanine given 15 min before sampling. n=5-16. Reference group compared to C26 using ANOVA; significance indicated by asterisk (*). C26-bearing groups compared using factorial ANOVA; significance indicated by uppercase letters for main effects (A: cys, B: ibu, C: interaction) and by different lowercase letters for between groups.

TABLE B.4 Free amino acids in tumor from mice bearing the colon 26 (C26) tumor fed a control or cystine (Cys) diet with and without ibuprofen (Ibu).

Mean ± SEM nmol/g	C26	C26+Cys	C26+Ibu	C26+Cys+Ibu
		Dietary Essential Amino Acids		
Histidine	437 ± 36	459 ± 43	457 ± 27	589 ± 93
Isoleucine	347 ± 41	327 ± 31	277 ± 19	349 ± 38
Leucine	710 ± 76	695 ± 60	600 ± 41	728 ± 72
Lysine	899 ± 58	1064 ± 89	832 ± 46	1063 ± 126
Methionine	354 ± 55	332 ± 35	354 ± 25	435 ± 57
Phenylalanine	1326 ± 91	1192 ± 155	1283 ± 82	1464 ± 130
Threonine	2017 ± 246	1997 ± 154	1881 ± 110	2174 ± 244
Tryptophan	272 ± 11[a,b]	261 ± 10[a,b]	225 ± 16[a]	297 ± 47[b]
Valine	941 ± 161	768 ± 85	694 ± 63	848 ± 113
		Dietary Non-Essential Amino Acids		
Alanine	1952 ± 87	1717 ± 150	1782 ± 105	2060 ± 98
Arginine	393 ± 18	427 ± 67	388 ± 35	480 ± 78
Asparagine	683 ± 40[a]	687 ± 72[a]	740 ± 30[a,b]	923 ± 92[b]

TABLE B.4 continued

Mean ± SEM nmol/g	C26	C26+Cys	C26+Ibu	C26+Cys+Ibu
Aspartate	397 ± 24	398 ± 42	376 ± 25	444 ± 27
Citrulline	542 ± 71	540 ± 86	419 ± 26	574 ± 85
Cyst(e)ine	382 30[a]	465 48[a,b]	429 62[a]	505 48[b]
Glutamate	3022 ± 151	2536 ± 290	2637 ± 141	3018 ± 170
Glutamine	554 ± 28[a,b]	508 ± 69[a]	585 ± 45[a,b]	713 ± 43[b]
Glycine[B]	3949 ± 188	3871 ± 440	4797 ± 309	5239 ± 627
Ornithine	191 ± 22	268 ± 63	184 ± 15	227 ± 35
Serine	497 ± 28	622 ± 91	540 ± 60	639 ± 74
Taurine[B]	5064 ± 70[a,b]	4776 ± 232[a]	5454 ± 95[b]	5678 ± 203[b]
Tyrosine	663 ± 57	624 ± 38	697 ± 36	787 ± 62

Note: Flooding dose of phenylalanine given 15 min before sampling. n=6-8. C26-bearing groups compared using factorial ANOVA; significance indicated by uppercase letters for main effects (A: cys, B: ibu, C: interaction) and by different lowercase letters for between groups.

TABLE B.5 Average daily intake of food, cyst(e)ine, methionine and total sulphur amino acids (SAA) in reference and colon 26(C26)-bearing mice fed either a control or cystine (Cys) supplemented diet with and without ibuprofen (Ibu).

Mean ± SEM	Reference	C26	C26+Cys	C26+Ibu	C26+Cys+Ibu
			g/kg/d		
Food	165.86 ± 8.32	157.28 ± 7.35	162.85 ± 6.41	163.01 ± 5.13	165.61 ± 4.20
Cyst(e)ine[A]	0.13 ± 0.01	0.12 ± 0.01[a]	2.27 ± 0.09[b]	0.12 ± 0.01[a]	2.31 ± 0.06[b]
Methionine	1.39 ± 0.07	1.32 ± 0.06	1.38 ± 0.05	1.37 ± 0.04	1.40 ± 0.04
Total SAA[A]	1.52 ± 0.08	1.45 ± 0.07[a]	3.65 ± 0.14[b]	1.50 ± 0.05[a]	3.71 ± 0.09[b]

Cyst(e)ine and methionine intake includes casein content and added free amino acids. n=10-16 cages per group. Reference group compared to C26 using ANOVA; significance indicated by asterisk (*). C26-bearing groups compared using factorial ANOVA; significance indicated by uppercase letters for main effects (A: cys, B: ibu, C: interaction) and by different lowercase letters for multiple group comparisons.